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THE UNIVERSITY OF ALBERTA

DOUBLE NUCLEOLAR ORGANIZING REGIONS AS A RISK FACTOR FOR DOWN SYNDROME

BY

ELIZABETH MARY ROBERTS

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF MASTER OF SCIENCE

DEPARTMENT OF GENETICS

EDMONTON, ALBERTA

(FALL,1988)

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Elizabeth J. Ives
(Supervisor)

Sept 21, 1988

Date: Sept 21, 1988

In memory of Norma Louise Francis Roberts

ABSTRACT

Jackson-Cook et al. (1985) presented data suggesting that the presence of double nucleolar organizing regions (dNORs) on acrocentric chromosomes predispose these chromosomes to nondisjunction and that carriers have a twenty-fold increase in risk for Trisomy 21 pregnancies. To assess the validity of the results and conclusions presented by Jackson-Cook et al. (1985), three groups of people were examined for dNOR(+) carriers. They included one hundred individuals that did not have a family history of nondisjunction (control group), twenty-five couples with one child trisomic for chromosome 21 (test group A), and five families in which three had more than one child with Down Syndrome and two had one child with Down Syndrome and another child with either Trisomy 13 or Trisomy X (test group B). A significant difference in the frequency of dNOR(+) carriers between the control group and test group A was not found. The results of test group B were inconclusive. Thus, it seems unlikely that dNORs are a factor in the nondisjunction of acrocentric chromosomes.

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I. INTRODUCTION

1. Down Syndrome: A distinct form of mental retardation.

Down Syndrome is one of the more common congenital disorders associated with mental retardation (Lilienfeld, 1969; Hall, 1964; Robinson and Puck, 1967). Those affected can easily be recognized by a general shortening of all parts of the body and various characteristic facial features including a protruding tongue, a broad flat face with a small nose, malformed ears, and close set eyes with narrow slanting eyelids (eg. Mange and Mange, 1980; Cohen and Nadler, 1983).

Despite the unique features of this disorder, Down Syndrome has only been recognized as a distinct form of mental retardation since the mid 1800's. The first to do so was John Langdon Down in a short paper published in the London Hospital Reports in 1866 (Down, 1866). Having established a home for mentally retarded children he began classifying their various congenital mental defects according to ethnic similarities. On the basis of the oriental-like slant of the eyes, Down Syndrome was referred to as a Mongolian type of idiocy. In 1876, unaware of Dr. Down's paper, John Fraser and Arthur Mitchell called attention to this disorder in a similar way by referring to it as Kalmuc idiocy (Fraser and Mitchell, 1876). The term was derived from the name of a Mongolian tribe found in the lower Volga region of Russia (the Kalmucks). In the years to follow these reports, several scientists around the world would also describe this disorder in a similar way (Jones, 1890; Oliver, 1891; Smith, 1896; Garrod, 1898). Although the terms Mongolian idiocy and Mongolism are obviously unscientific and offensive, they surprisingly remained the common designation of this disorder for more than a century. Only recently have they been replaced by the terms Down's Syndrome and Down Syndrome (Volpe, 1986).

Almost a century after Dr.Down's paper was published, the field of human genetics had reached a point where one could begin to understand the cause(s) of Down Syndrome. After it had been established that the normal number of chromosomes in a human somatic cell is 46 (See Figure 1) (Tjio and Levan, 1956; Ford and Hamerton, 1956) several cytogeneticists had reported that individuals with Down Syndrome carried 47 chromosomes (Lejeune et al., 1959; Jacobs et al., 1959; Ford et al., 1959; Book et al., 1959). Initially, it was thought that the extra chromosome was the larger of the two G-group chromosomes. According to the standard nomenclature of the 1960 Denver Conference this would be chromosome No. 21 (International Study Group, 1960). For this reason Trisomy 21 was accepted as an alternate name for the disorder. However with improved techniques in cytogenetics (Paris Conference, 1971), it was discovered that the extra chromosome was in fact the smallest of the G chromosomes (Caspersson et al., 1970a). Rather than changing the name to Trisomy 22, cytogeneticists simply accepted the inconsistency and continued to refer to the smallest G-group chromosome as No. 21 (See Figure 2) (Mange and Mange, 1980).

Subsequently it was discovered that (1) Down Syndrome can be the result of a translocation (eg. t(14q21q)) (Polani et al., 1960; Fraccaro et al., 1960) and (2) some affected individuals may be chromosomal and genetic mosaics with two cell populations; one with a normal chromosome number and the other with cells trisomic for chromosome 21 (Clarke et al., 1961; Blank et al., 1962). These situations will however not be discussed in this thesis.

2. The Frequency of Trisomy 21.

Among Caucasians, the frequency of Down Syndrome is approximately 0.15 percent in the general population (Hook, 1978). The possibility of a maternal and/or

FIGURE 1. Solid stained (Giemsa) karyotype of a normal human female

The chromosomes in this karyotype are from a female human lymphocyte in metaphase. The chromosomes were paired (where possible) and arranged in order of decreasing size on the basis of length and centromere position. They were also classified into seven distinct groups designated A to G (International Study Group, 1960).

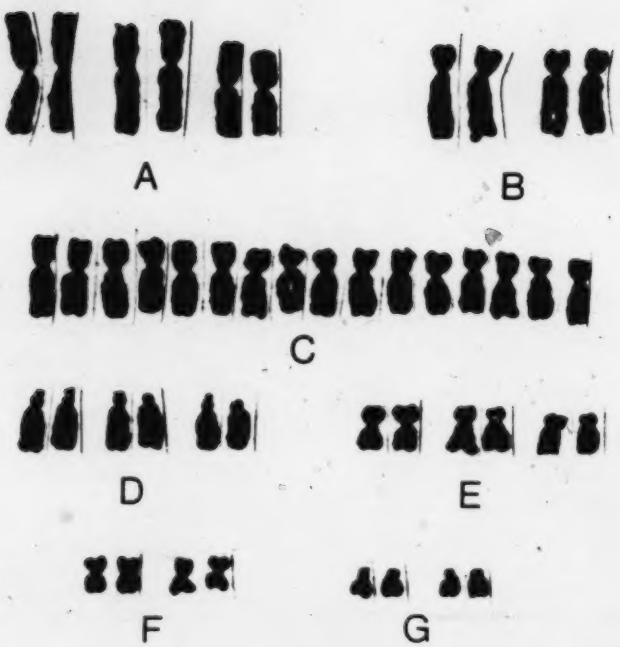

FIGURE 1. Solid stained (Giemsa) karyotype of a normal human female

FIGURE 2. G-banded karyotype of a human female trisomic for chromosome 21

The 47 chromosomes were paired and classified into seven groups on the basis of their G-banding patterns (chromosomes 1, 2, and 3 in group A; chromosomes 4 and 5 in group B; chromosomes 6 to 12 and, although shown separately, X in group C; chromosomes 13, 14, and 15 in group D; chromosomes 16, 17, and 18 in group E; chromosomes 19 and 20 in group F; and chromosomes 21 and 22 in group G) (Paris Conference, 1972).

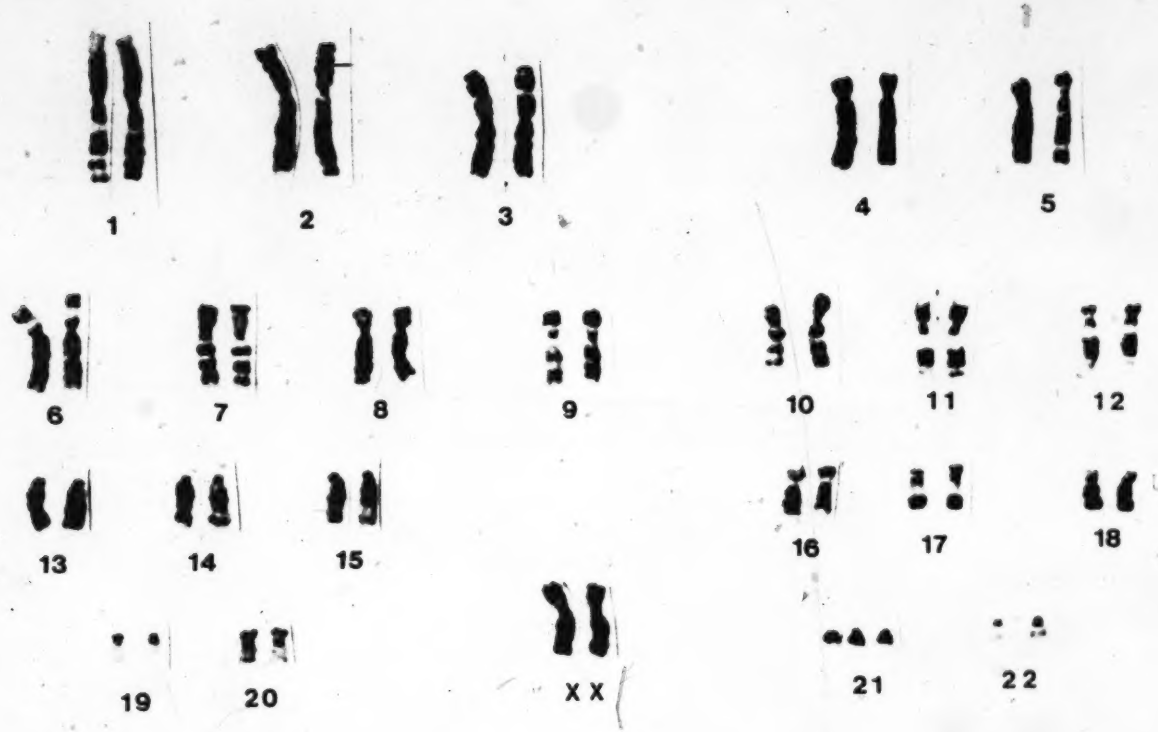

FIGURE 2. G-banded karyotype of a human female trisomic for chromosome 21

paternal age effect had been suggested as early as 1876 with the observation that most affected individuals were among the last-born members of a sibship (Fraser and Mitchell, 1876). By the 1930's, epidemiological studies had shown that while mothers over the age of thirty-five gave birth to only fifteen percent of all live births they had produced over fifty percent of all Down Syndrome babies (eg. Jenkins, 1933; Penrose, 1967).

In a more recent report the estimated rate of Down Syndrome for various maternal ages was calculated. As shown in Figure 3, the estimated risk of a woman producing an affected child is quite low at approximately twenty years of age. While this value will increase only slightly up to thirty-five years it will rise dramatically beyond this age level (Hook and Chambers, 1977).

The father's age may also be a causal factor in nondisjunction but unfortunately the few studies which have investigated this possibility have produced inconclusive results (eg. Erickson and Bjerkedal, 1981; Hook and Regal, 1984).

For the parents of a child with Trisomy 21, the recurrence risk has been estimated at 1.0 to 1.3 percent (Daniel et al., 1982). Like the parents, second degree relatives (ie. aunts, uncles, nephews, and nieces) and third degree relatives (ie. first cousins, greataunts, and greatuncles) of a proband may also be at an increased risk of having an affected child. Unfortunately as shown in Table 1, the magnitude of this risk has not been estimated to anyone's satisfaction. While some believe it to be approaching the general population's frequency of 0.15 percent (Abuelo et al., 1986; Eunpu et al., 1986), others believe it to be closer to 1.0 percent (Hecht et al., 1983; Tamaren et al., 1983).

3. The Origin of Nondisjunction Leading to Trisomy 21.

Most cases of Trisomy 21 may be traced back to nondisjunction during meiosis in either the male or female parent (Lejeune et al., 1959; Hamerton, 1971). Through

FIGURE 3. Estimated rate of Down Syndrome for different maternal ages

For twenty year old mothers the risk of having a Down Syndrome child is one in 1925 births. Although this value will increase only slightly up to the maternal age of thirty-five years (ie. one in 365 births) it will rise dramatically beyond this age level (From the figures of Hook and Chambers, 1977).

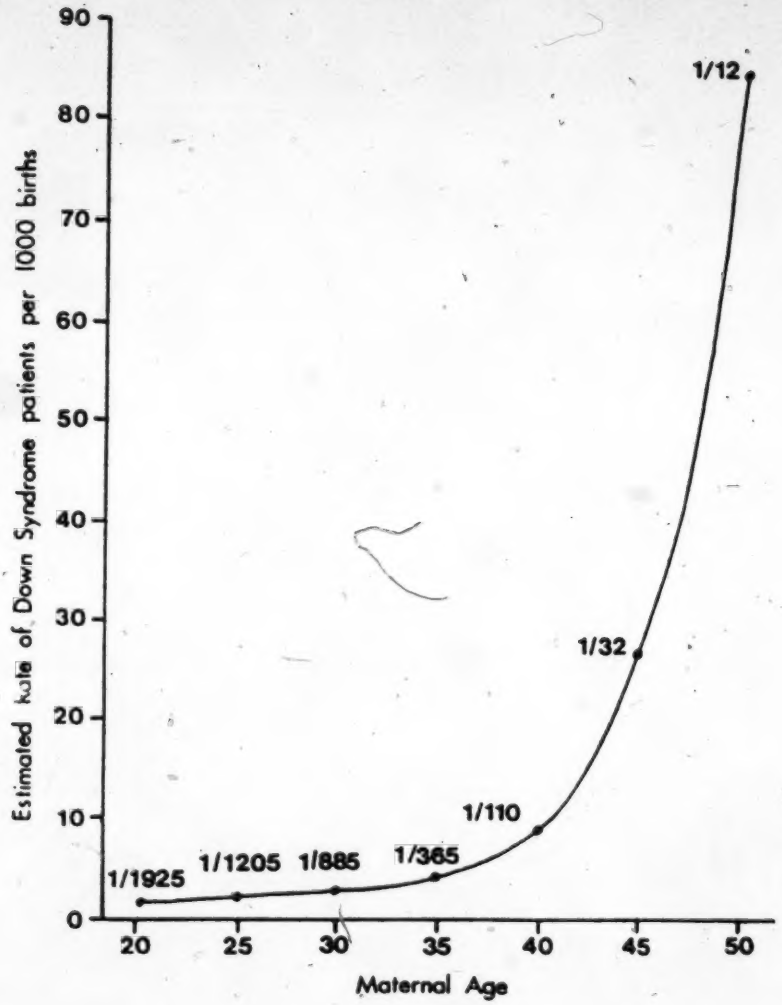

FIGURE 3. Estimated rate of Down Syndrome for different maternal ages

TABLE 1

Risk of Down Syndrome in Relatives

Relationship to the proband	Percent affected			
	Hecht et al. (1983)	Tamaren et al. (1983)	Abuelo et al. (1986)	Eunpu et al. (1986)
First degree (parents and siblings)	1.65%	1.65%	0.38%	0.95%
Second degree (nieces, nephews, aunts, and uncles)	0.65%	0.67% *	0.12%	0.00% *
Third degree (first cousins, great aunts, and great uncles)	0.26%	0.56% *	0.08%	/
General population	0.15%	0.18%	0.08%	0.08%

* Maternal age ≤ 35 years.

quinacrine staining, the Q band polymorphism (Caspersson et al., 1970b) of chromosome 21 has made it possible to study the origin of this meiotic error with respect to both the parent and the stage of meiotic division (Robinson, 1973). Based on the results of Manning and Goodman (1981), del Mazo et al.(1982),and Juberg and Mowrey (1983), it would appear that most cases of meiotic nondisjunction are of maternal origin occurring during the first division of meiosis. For example, Juberg and Mowrey (1983) found that the ratio of first division to second division errors during meiosis was four to one and three to two among the maternal and paternal cases respectively. In addition, they also reported that maternal origin far exceeded that of paternal origin, accounting for eighty percent of all trisomic cases.

4. Causes of Meiotic Nondisjunction.

(a) Reduced rate of crossing over in chromosome 21 bivalents.

There have been a number of papers published which offer possible explanations for the cause(s) of meiotic nondisjunction. Antonarakis et al. (1986) and Warren et al. (1987) favoured the hypothesis that nondisjunction is the result of a reduced rate of crossing over in chromosome 21 bivalents. This was prompted by (1) the hypothesis that the orderly segregation of homologous chromosomes during anaphase I requires at least one chiasma per bivalent (Mather, 1938) and (2) studies on mice which had shown that nondisjunction is usually due to the abnormal segregation of univalents at anaphase I (Henderson and Edwards, 1968) (The univalents are produced by either a failure of any chiasma formation between homologues (asynapsis) or a premature separation of homologues following normal chiasma formation (desynapsis) (Beadle, 1930; Li et al., 1945; Soost, 1951)).

Believing that nondisjunction during the first meiotic division is caused by the unequal segregation of univalents produced by asynapsis, Antonarakis et al. (1986) wanted to test this hypothesis by examining chiasmata on chromosomes 21 that had undergone nondisjunction. Since this was not possible they chose to study genetic recombination among DNA markers on these chromosomes. Given evidence that chiasma formation and genetic recombination are directly related (Beadle, 1932; Jones, 1971; Polani and Crolla, 1982), Trisomy 21 due to asynapsis was therefore considered equivalent to no recombination. Consequently, to test whether asynapsis had occurred on chromosomes 21 that had undergone nondisjunction, the authors estimated the recombination frequencies between the centromere and marker loci on chromosomes 21 that had undergone nondisjunction and on those that had disjoined normally. The data showed that recombination was reduced on the nondisjoined chromosomes 21 relative to those that had disjoined normally (See Table 2). These results indicated to Antonarakis et al. (1986) that asynapsis is an important factor in meiotic nondisjunction leading to Trisomy 21 in humans.

It is not known whether the observed reduction in recombination in Trisomy 21 families occurs only in chromosome 21 bivalents. If it is limited to this chromosome then perhaps chromosome-specific gene(s) exist which control crossing over during pachytene. This would explain the results of Antonarakis et al. (1985). Attempting to test the hypothesis that there is a genetic predisposition to Trisomy 21 associated with DNA sequences on chromosome 21, Antonarakis et al. (1985) used DNA polymorphism haplotypes for this chromosome to examine the distribution of different chromosomes 21 in control families and in families with a Down Syndrome child from the same ethnic group. They found three different haplotypes accounted for the majority of chromosomes 21 in the control families. A different haplotype was found to be more common among the chromosomes 21 that had undergone nondisjunction in the families with a Down Syndrome child. From these observations the authors proposed that a subpopulation of chromosomes

TABLE 2

Estimation of the recombination frequencies between the centromere and the marker loci on chromosomes 21 that undergo nondisjunction ($\hat{\theta}_T$) and on those that disjoin normally ($\hat{\theta}_C$).

Marker loci*	$\hat{\theta}_T$	$\hat{\theta}_C$	$\hat{Z}(\theta_T, \theta_C)$	χ^{2}**	P-Value
CW21PC-D21S13	0.00	0.30	1.23	0.61	0.22
CW21PC-D21S1/D21S11	0.00	0.20	2.67	3.01	0.04
CW21PC-D21S3/D21S23	0.00	0.25	1.95	2.92	0.04

* Note:
1. Marker CW21PC is a pericentromeric marker found on the long arm of chromosome 21. It is present in single copy.
2. Markers D21S13, D21S1/D21S11, and D21S3/D21S23 also map to the long arm of chromosome 21 and are present in single copy.
3. The LOD score is $Z(\theta, y)=\log_{10}[L(\theta, y)/L(0.5, 0.67)]$
4. For a description of the method used to calculate θ_T, θ_C, and Z see Antonarakis et al. (1986) and Warren et al. (1987).

**for $\theta_T = \theta_C$ vs. $\theta_T < \theta_C$

21 with this haplotype carry DNA sequences with an increased tendency for nondisjunction leading to Trisomy 21

(b) Malfunction of the spindle system.

There have been reports of elevated aneuploidy in the mitotic cells of individuals prone to meiotic nondisjunction (eg. parents of Down Syndrome children) (eg. Juberg et al., 1985). This would suggest that the factors involved in nondisjunction are perhaps operating concurrently in the meiotic and mitotic cells. Therefore by studying the behavior of chromosomes in mitotic cells from individuals prone to meiotic nondisjunction, one should gain a better understanding of their behaviour in meiocytes. This line of reasoning was used by Dotan and Avivi (1986) in a study that examined the possibility of meiotic nondisjunction resulting from a malfunction of the spindle system. Based on their risk of Trisomy 21 pregnancies, three groups of women were chosen for their study: (1) a low risk group of women twenty-two to thirty-four years old, (2) a high risk group of women twenty-six to thirty-three years old who gave birth to children with Down Syndrome while in their early twenties, and (3) a high risk group of women forty to fifty-two years old. In each case mitotic cells were exposed to various antimicrotubule drugs and examined for their spindle sensitivity (Spindle sensitity was expressed as the percentage of fully arrested metaphases out of the total metaphase cell population, ie., cells exhibiting short, thick, and condensed chromosomes.). As the concentration of each drug was increased, the authors found that the women of the low risk group exhibited a significantly higher spindle sensitivity than those of the two high risk groups. The observed differences in mitotic microtubule properties between the women of the low and high risk groups led the authors to conclude that an involvement of the spindle system in meiotic, as well as mitotic, nondisjunction may exist.

The idea that factors involved in meiotic nondisjunction are working concurrently in mitotic cells made Dotan and Avivi (1986) suspicious of the theory that assumes the high

frequency of meiotic nondisjunction in women over the age of thirty-five is due to a gradual accumulation of anomalies in the oocyte during the many years that it has been in prophase I. The authors therefore suggest that a female's biological age (ie. her proximity to menopause) is more significant than her chronological age in her risk of meiotic nondisjunction. This would imply that some of the same hormonal factors that are involved in the onset of menopause may play a role in the malfunction of the spindle system leading to meiotic nondisjunction. How would this explain the observation that the mitotic cells of young Down Syndrome mothers responded to the antimicrotubule drugs in the same way as those of older women? The authors suggest that perhaps an acceleration of a woman's biological age is possible. The cause of this may be genetic, environmental, physiological, or a combination of the three.

The concept of a hormonal influence on meiotic nondisjunction has been considered by other authors (eg. Hansmann and Jenderny, 1983; Hansmann et al., 1985; Golbus, 1983). According to Hansmann et al. (1985) not only are the germ cells physically surrounded by somatic cells during most stages of meiosis, but they are also metabolically coupled with them in such a way that a failure in endocrine control can indirectly lead to aneuploidy. Working with female Djungarian hamsters, the authors found that following an injection of gonadotrophins into the animal, a significant number of the oocytes examined were aneuploid; this number increased with increasing doses of the hormone. These workers were unable to explain how the gonadotrophins act on the oocyte since it does not appear to have specific receptor sites for the hormone. They suggest that the effects are most likely mediated in some way through the somatic cells surrounding the germ cells. It was also noted that the last forty-eight hours of follicular differentiation appear to be the most crucial time for meiotic errors to occur. Hansmann et al. (1985) suggest that the sudden introduction of gonadotropins has interfered with the normal preovulatory development of the somatic cells surrounding the ovum, thereby disturbing its ripening and allowing nondisjunction to occur.

(c) Centromere separation during second meiotic division.

While the majority of cases of meiotic nondisjunction seem to occur during the first meiotic division, errors during the second meiotic division have also been detected. One proposal yet to be tested centers around centromere separation in man (eg. Vig, 1984; Fitzgerald et al., 1986). If, as stated by Vig (1984), sister chromatids were to separate too early the centromeres may not be "mature" enough to receive the spindle fibers for proper attachment. As well, if there was a delay in chromatid separation the centromeres may not separate in time to coincide with spindle activity. The author suggests that this proposal could explain why certain chromosomes appear to be more prevalent in cases of aneuploidy given the increasingly accepted view that chromosomes in a given genome separate in a nonrandom and sequential manner (Vig, 1981; Vig and Woinicki, 1974; Mehes, 1975). Human chromosome 18, for example, appears to be one of the first chromosomes to undergo centromere separation. The acrocentric chromosomes (ie. 13, 14, 15, 21 and 22) appear to be the last to separate. Consequently, if the chromatids of chromosome 18 were to separate early or those of any of the acrocentric chromosomes were to separate late, aneuploidy could result. Chromosomes which normally separate in the middle of the sequence would most likely not be affected by premature or delayed separation because, assuming that the spindle fibers become available to all centromeres at the same time, the spindle-centromere attachments would have already taken place.

(d) The nucleolar organizing region's role in nondisjunction.

Of all the chromosomes, No. 21 is the one most commonly involved in nondisjunction (eg. Verma et al.,1986). Like all acrocentrics in man, No. 21 has a short p arm consisting of the secondary constriction and the terminal satellite. It is within the secondary constriction that the nucleolar organizing region (NOR) is located. During interphase, and persisting through most of prophase I, this is the site of association

between the chromosome and the nucleolus (Ohno et al., 1961; Ferguson-Smith and Handmaker, 1961). Studies have shown that the NORs contain the 18S and 28S ribosomal RNA (rRNA) genes (Ritossa and Spiegelman, 1965). There are 400 copies of these genes per genome distributed among the five acrocentric chromosomes (Bross and Krone, 1972). Their number per chromosome varies from one chromosome to another and from one person to another (eg. Dittes et al., 1975).

When the NORs are silver stained (eg. Goodpasture and Bloom, 1975), the amount of silver stained material is not consistent among the acrocentric chromosomes (eg. Miller et al., 1977) (Also see Figure 4). It seems likely that some of this variation is due to differences in the amount of ribosomal DNA (rDNA) (Evans et al., 1974; Henderson et al., 1972; Warburton et al., 1976). However this cannot explain why unstained NORs are frequently found (eg. Miller et al., 1977) given that all normal acrocentric chromosomes possess copies of rRNA genes (Bross and Krone, 1972). Recent studies have shown that an NOR's silver stainability is actually a reflection of its transcriptional activity in the preceding interphase rather than merely the presence of the rRNA genes (D.A. Miller et al., 1976; O.J. Miller et al., 1976; Engel et al., 1977; Hansmann et al., 1978; Schmid et al., 1977). Present evidence suggests that the silver stained material consists of non-histone acidic proteins (Howell et al., 1975; Goodpasture and Bloom, 1975; Howell, 1977). These proteins carry sulfhydryl and disulphide groups which react with Ag^+ ions to produce the characteristic black spot of precipitated metallic silver on the NOR (Buys and Osinga, 1980). In the somatic mitoses, residues of these proteins tend to remain associated with the NORs into metaphase and can thus be silver stained at this stage even though the NORs are now transcriptionally inactive (Fan and Penman, 1971). The role(s) of these NOR-associated proteins are not known. Some studies suggest that they are rDNA binding proteins (Likovsky and Smetana, 1981; Olson and Thompson, 1983; Olson et al., 1983). Other evidence indicates that they may be associated with ribosome maturation

FIGURE 4. Male human lymphocyte at metaphase as it appears after silver staining

The amount of silver stained material on each NOR varies from one acrocentric chromosome to another. An NOR may be heavily stained (a, c, e, and f), lightly stained (b and d), or show no stain (g). Only six of the ten acrocentric chromosomes are silver stained in this cell. One of the acrocentric chromosomes in this figure has a silver stained double NOR (b).

FIGURE 4. Male human lymphocyte at metaphase as it appears after silver staining

(Bourbon et al., 1983). Williams et al. (1982) has even suggested that the silver staining proteins are associated with RNA polymerase I.

In both mitotic and meiotic cells, the NORs of two or more acrocentric chromosomes can be found in close contact with one another in what is called a satellite association (Ferguson-Smith and Handmaker, 1961; Mirre et al., 1980; Schmid et al., 1983). This relationship reflects the participation of the acrocentric chromosomes in the formation of the nucleolus. It has been speculated by Mirre et al. (1980) and Verma et al. (1986) that such close contact of the acrocentric chromosomes may lead to errors in chromosome pairing or separation. While the exact mechanism of this is not known, these authors favour the following hypothesis: Until ovulation human oocytes are present at dictyotene. Since the nucleolus is still present at this stage, paired acrocentric chromosomes which have participated in its formation will remain in an association of the NORs for approximately twelve to fifty years. Perhaps the NOR association is reinforced by the formation of a protein matrix. Although normally degraded by an enzyme before metaphase I, the protein matrix may persist beyond this point if, after an extended period of time, the enzyme is no longer functional or in a high enough concentration to act against the protein.

Direct evidence that the association of NORs is a factor in nondisjunction has been difficult to obtain. While some studies have shown that the frequency of satellite associations is higher among the parents of trisomic offspring than it is among control couples (Mattei et al., 1974; Hansson, 1979), other studies refute this claim (Cooke and Curtis, 1974; Taysi, 1975; Jacobs and Mayer, 1981). Perhaps only some NORs, and the satellite associations they form, are responsible for meiotic nondisjunction. For example, Verma et al.(1986) has found that among the parents of Down Syndrome offspring, the parent who contributed the extra chromosome 21 had, in all cases, active ribosomal cistrons (ie. silver stained NORs) on both chromosomes 21. While as yet the authors have not examined a control population they believe that their data indicates that only satellite

associations between two chromosomes with active NORs will lead to meiotic nondisjunction.

Jackson-Cook et al. (1985) were also interested in a possible role of the NOR in the etiology of nondisjunction. They presented data suggesting a strong association between a specific variant of the NOR and Trisomy 21. This variant is described as a doubling or duplication of the NOR which may involve any of the acrocentric chromosomes and has been termed a double NOR (dNOR) (Archidiacona et al., 1977) (For an example of a dNOR see Figure 4).

How the dNOR is formed is not known. Some studies suggest that it is the result of a translocation in which the satellite and part of the short arm on one acrocentric chromosome are translocated on to another acrocentric chromosome which in turn has lost either part or all of its satellite (Rocchi et al., 1971; Bauchinger and Schmid, 1970).

Schmickel et al. (1985) suggest that an acrocentric chromosome with a dNOR is the result of a Robertsonian translocation. In general the two non-homologous acrocentric chromosomes involved in a Robertsonian translocation break near their centromeres (In one chromosome the break occurs on the long arm while in the other chromosome the break occurs on the short arm.). The two chromosomes mutually exchange their acentric segments to form two new metacentric chromsomes (one large chromosome with all of the essential genes and one short chromosome composed of heterochromatin) (Hamerton, 1966). However, if the breaks were to occur on the short arm of each acrocentric chromosome, distal to their centromeres, the Robertsonian translocation could lead to the formation of a large dicentric chromosome with two NORs. A monocentric chromosome with two NORs (ie. dNOR) could therefore be the result of anaphase breakage after the dicentric chromosome had been formed.

Lau et al. (1979) studied a 14p+ variant by means of silver staining and N-banding. They observed that only certain regions of the p+ arm were stained with the silver. The authors also noted that the number of silver stained regions varied on the p+ arm from zero

to three among cells from the same blood culture. In contrast to these observations, the authors found that with N-banding, a staining method that is also specific to the NOR (Matsui and Sasaki, 1973), the entire short arm of the chromosome was stained. Lau et al. (1979) also performed in situ hybridization experiments with rat 18S ^{125}I-rRNA on cells from the individual with the marker chromosome. They found labeling to be located along the entire p+ segment of this chromsome. The N-banding and in situ hybridization experiments suggested to the authors that the p+ arm had only one large NOR rather than two or three as indicated by silver staining. Since silver staining reacts only to rDNA sites that are transcriptionally active (eg. D.A. Miller et al., 1976; Engel et al., 1977), Lau et al. (1979) attributed the variability in the number of staining regions exhibited by this method to a fluctuation in the regulation of rRNA gene activity along the NOR. From the in situ hybridization study the grain counts indicated that the p+ segment contained at least eight times more rDNA than that of a normal acrocentric chromosome. The authors believed that this large NOR was the result of a tandem duplication or amplification of the rDNA.

Figure 5 was taken from Figure 1 of Jackson-Cook et al. (1985). According to these workers a dNOR can vary in its appearance from two completely separate areas of silver staining (one proximal and one distal to the centromere) (eg. chromosome 15) to a single dumbbell shaped area of silver staining (eg. chromosome 13).

Based on this level of classification the authors found that in fifteen of fifty couples with a child trisomic for chromosome 21 at least one parent carried a dNOR on one of the acrocentric chromosomes (In one couple both parents were dNOR(+) carriers). In contrast, this variant was not found in any of the fifty control subjects. In thirteen of the fifteen couples with at least one dNOR(+) carrier, Jackson-Cook et al. (1985) were able to determine the parental origin of nondisjunction through Q-banding heteromorphisms. They found that in all cases, the parent carrying the dNOR was also the source of the meiotic error. They also observed that in eleven of the thirteen informative dNOR couples the nondisjunction had occurred during the first meiotic division.

FIGURE 5. Appearance of the double NOR variant chromosomes

The fifteen acrocentric chromosomes with dNORs as they appear with QFQ staining (dark column) and silver, or NOR, staining (light column). The dNORs may appear as two completely separated regions (as in the chromosome 15 at the bottom of this figure) or as two somewhat confluent regions (as seen in the other chromosomes) depending on chromosome morphology and the nature of the staining reaction. The dNOR variants are also characterized by elongated stalks as seen in the QFQ staining of these chromosomes (From Figure 1 of Jackson-Cook et al., 1985).

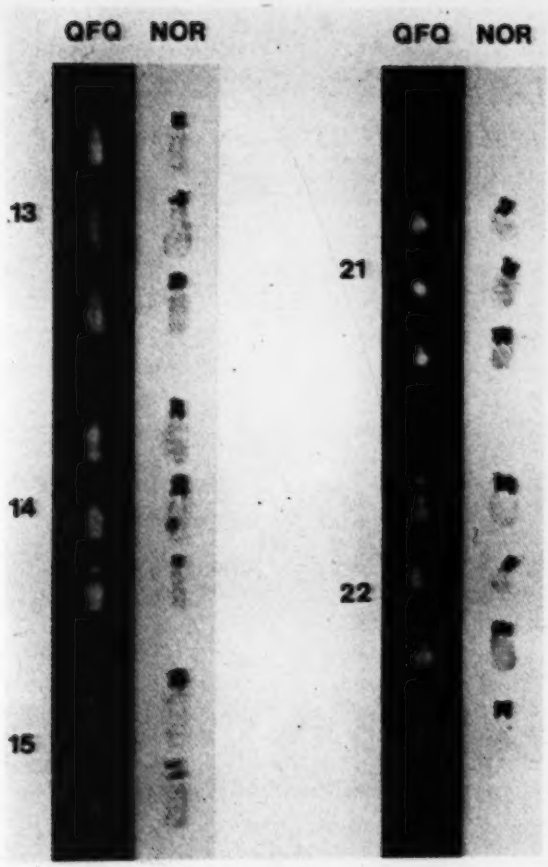

FIGURE 5. Appearance of the double NOR variant chromosomes

Based on their results, Jackson-Cook et al. (1985) proposed that in germ cells acrocentric chromosomes with dNORs may possess extra binding sites that promote satellite associations between nonhomologues that may contribute to nondisjunction. The authors noted that this may occur more often in females because of the extensive period of time in which such associations may exist. The authors conclude that if dNORs are a factor in nondisjunction then the data suggests that carriers have as much as a twentyfold increase in risk for Trisomy 21 pregnancies.

5. The Project.

Based on the work cited several explanations have been proposed to account for nondisjunction leading to Trisomy 21. Whether any or all of them are correct is still unknown. I will be examining in detail the proposal made by Jackson-Cook et al. (1985) that dNORs may increase the risk of having a child with Trisomy 21.

It is important to examine this proposal to determine whether it is a plausible mechanism of the origin of aneuploidy. A number of cytogeneticists have attempted to duplicate their results as well as add to them with an examination of the frequency of dNORs in the general population. Although certain research groups have produced data which they believe supports a dNOR-nondisjunction relationship (eg. Jones et al., 1987; Melnyk et al.,1987), many others have produced negative results (eg. Hassold, 1987; Hassold et al., 1987; Patil, 1987; Soukup, 1987; Kousseff, 1986; Goodwin and Kousseff, 1986; Spinner et al., 1986; Spinner, 1987).

To test the proposal made by Jackson-Cook et al. (1985), I have examined three groups of people for dNOR(+) carriers. They included (1) a control group consisting of one hundred individuals that did not have a family history of nondisjunction, (2) twenty-five couples with one child trisomic for chromosome 21 and no other family history of

nondisjunction (test group A), and (3) five families in which, within the recorded pedigree, three had more than one child with Down Syndrome and two had one child with Down Syndrome and one child with either Trisomy 13 or Trisomy X (test group B).

II. Materials and Methods

1. Ethic's Committee Approval.

Given that the project required the use of human subjects, an outline of the study was presented to the University of Alberta Faculty of Medicine's Ethic's Committee. The project was approved by this committee under the conditions that (1) each participant would be given a verbal and written explanation of his, or her, role in the study (See Appendix 1 and 2), (2) each participant would be required to read and sign a consent form for the collection of blood samples (See Appendix 3), and (3) upon the completion of the study, all results would be made available to the participants with an explanation of their meaning in either a verbal or written form.

2. Choosing the Human Subjects for Test Group A and Test Group B.

(a) Compiling a list of individuals with Down Syndrome.

A list of names was compiled from the Edmonton Genetics Clinic (EGC) that represented all of the cases of Down Syndrome (including fetuses, newborns, and children) which had (1) either directly or indirectly been in contact with the EGC from the beginning of 1980 to the end of 1987 and (2) been diagnosed as 47,XX,+21 or 47,XY,+21 rather than as mosaics or translocation Down Syndrome individuals.

(b) Sources of information.

The EGC, which serves Northern Alberta, is made aware of individuals with Down Syndrome through a number of sources, For example, whenever the University of Alberta Hospitals' Cytogenetics Laboratory receives a blood or amniotic fluid sample for chromosome analysis, a copy of the requisition form and the results of the chromosome

analysis are filed in the EGC. A second source of information comes from the community component of the Alberta Hereditary Disease Program. The majority of Down Syndrome individuals in Alberta are well known to the outreach nurses in this program through whom they are receiving services. As in the case of this research project, the outreach nurses are commonly asked to provide information on these individuals (eg. family history). Another obvious source of information comes from the genetic counselling service the EGC offers to the public. This facility has served a number of parents, as well as other relatives, of Down Syndrome children seeking counselling on their risk of having additional children affected with this disorder.

(c) Examining the EGC's patient files.

Since the project dealt with determining if carriers of dNORs are at risk in producing Trisomy 21 offspring, the list of affected children was used to compile a list of couples thought to be suitable for either test group A or test group B. To develop this second list required access to the EGC's patient files. This was made available under the supervision of Dr. E.J. Ives. The information stored in these files was found to range from a single requisition for, and the chromosome analysis of, a blood or amniotic fluid sample to a complete documentation of family history, patient and relatives' names, addresses and dates of birth, as well as the names and addresses of referring health personnel.

(d) The criteria for choosing human subjects.

The criteria for placing a couple in either test group A or test group B included (1) a confirmation of trisomy 21 in the proband through the chromosome analysis of either a blood or amniotic fluid sample, (2) a maternal age of less than thirty-six years, (3) both parents of the affected child being alive and residing in Alberta, (4) a current phone number

and address in order that the family may be contacted, and (5) documentation of family history as far back as the proband's third degree relatives.

If the first three criteria were not met the couple was omitted from the study. However, if the last two criteria were not met an attempt was made to obtain the necessary information. In most instances the health personnel that had referred the couple and/or the affected child to the EGC were able to provide the missing information from their own records or at least give permission to allow direct contact with the family.

(e) Contacting the families.

Having obtained a list of potential couples that had fulfilled the criteria specified for either test group A or test group B the next step was to contact them, explain the project and, if they were agreeable, obtain the blood samples needed for the chromosome analysis. In some instances the only person who had had direct contact with the EGC was a second or third degree relative or the physician of the proband. In this situation it was appropriate to notify them first, explain the project, and ask that they approach the family (or families) or give permission for the EGC to do this directly.

Eventually all of the couples of interest were contacted and given time to consider the proposed study, ask questions, and notify the EGC with a final decision. Couples that had agreed to participate were asked to have the blood samples taken at the University of Alberta Hospitals' Outpatient Laboratory or at a clinic of their choice. The blood samples, along with the consent and blood requisition forms, were then sent to the University of Alberta Hospitals' Data Center from where they were delivered to the Cytogenetics Laboratory for processing.

3. The Control Group.

Due to (1) the limitations of time and resources available to this research project and (2) the large sample size needed to make the analysis of the control group significant, it was not feasible to actually select one hundred normal individuals from the general population for blood to be drawn and processed for chromosome analysis. Instead, the University of Alberta Hospitals' Cytogenetics Laboratory was used as a source of control subjects by way of its large supply of unstained slides.

With every blood sample that is sent to this facility for chromosome analysis, approximately sixteen slides per blood sample are made. Of this number, only a few are actually needed for the standard analysis (eg. G-banding). Therefore at least four slides are left unstained and stored for the possibility of future use (eg. Q-banding or the silver staining of NORs).

Many of the blood samples sent to the cytogenetics laboratory are taken from chromosomally normal individuals or individuals with chromosomal abnormalities other than trisomies (eg. deletions, translocations). These samples are mainly sent to the laboratory for chromosome analysis because the patient, the patient's physician, or the patient's family wishes to determine if the patient's physical and/or mental defects are due to a chromosomal abnormality.

Since the objective of this study was to determine whether a dNOR has a role in meiotic nondisjunction, individuals that were (1) either chromosomally normal or had a chromosomal abnormality other than trisomies and (2) did not have a family history of nondisjunction were considered acceptable as subjects in the control group. Therefore prepared slides (approximately two slides per subject) from one hundred individuals fulfilling these criteria were taken from the University of Alberta Hospitals' Cytogenetics Laboratory for NOR analysis. Subjects were also chosen on the basis that (1) the slides

were made in 1987 so that their staining quality would be acceptable for this analysis and (2) the distribution of male and female subjects was approximately equal.

For each subject that proved to be a dNOR(+) carrier his family history was rechecked for evidence of meiotic nondisjunction. Often this required contacting the physician who had requisitioned the blood sample for chromosome analysis and asking for their permission to contact the subject and/or the family. All dNOR(+) carriers with a family history of nondisjunction were omitted from the normal control group.

4. Culturing and Harvesting the Blood Samples.

All of the blood cultures were established and later harvested according to the protocols used in the University of Alberta Hospitals' Cytogenetics Laboratory (Rothfels and Siminovitch, 1958; Arakaki and Sparkes, 1963).

(a) Collection of blood samples.

Approximately 10 ml. of venous blood was collected in a heparinized vacutainer from each subject. The blood was put in culture within twenty-four hours of being drawn.

(b) Materials.

1. sterile 30 ml. bottles with screw on caps
2. 37°C, 5% CO_2 incubator
3. laminar flow hood
4. graduated centrifuge tubes
5. frosted ended slides stored in ice water
6. sterile pasteur, graduated, and micropipettes
7. sterile syringes
8. sterile needles

9. sterile filtering system
10. slide warmer
11. centrifuge
12. 37°C waterbath
13. photomicroscope

(c) Solutions and reagents.

1. RPMI (1640) (Gibco) powdered
2. Fetal Calf Serum (Gibco) (FCS) -heat activated, mycoplasma and virus screened.
3. Phytohemagglutinin-M (Difco) (PHA)
4. Penicillin and Streptomycin (Gibco) (p+s) -10,000 units, lyophilized
5. deionized water
6. glacial acetic acid
7. absolute methanol
8. colcemid (Gibco) -lyophilized
9. potassium chloride
10. heparin (1,000 usp units/ml.)
11. sodium bicarbonate

(d) Stock solutions

1. RPMI

Found in a powdered form, RPMI was dissolved in water and then sterilized by filtration. After adding p+s (5 ml. per 1000 ml. of the medium) the RPMI was stored at 4°C for up to four weeks.

2. Culture medium

For each culture, 0.5 ml. of FCS was mixed with 4.5 ml. of stock RPMI.

3. Hypotonic solution (0.075M KCl with 16 units/ml. of heparin)

The hypotonic solution was made by mixing 2.74 gm. of KCl with 500 ml. of deionized water and 8.0 ml. of heparin (1000 usp units/ml.). The solution was stored at 4°C.

4. Fixative

Fixative was made by mixing three parts methanol to one part glacial acetic acid. Fresh fixative was made each time it was needed since on standing it absorbs water and changes pH.

(e) Method for establishing and harvesting the blood cultures.

Working under a laminar flow hood, a sterile cotton plugged pasteur pipette was used to place approximately 0.25 ml. (ie. five to ten drops) of venous blood into a sterile 30 ml. bottle containing 5.0 ml. of culture medium and 0.1 to 0.15 ml. of PHA (Four cultures for each blood sample were set up in this manner.). After gently shaking the cultures, they were immediately placed in a 5% CO_2 incubator at 37°C for seventy-two to ninety-six hours. During this time the bottle caps were left unscrewed to allow for proper gas and moisture exchange. Between thirty and forty-five minutes before harvesting the cultures, 0.15 ml. of colcemid was added to each bottle.

To harvest the blood cultures, the contents of each bottle were transferred to a centrifuge tube and spun at 1000 r.p.m. for ten minutes. Following this, the supernatant was removed and replaced with approximately 5.0 ml. of prewarmed hypotonic solution. Each suspension was placed in a 37°C waterbath for seven to ten minutes and then spun at

1000 r.p.m. for ten minutes. Next, the supernatant was removed and replaced with approximately 5.0 ml. of freshly prepared fixative. To avoid the formation of clumps that can be difficult to disperse, the fixative was added slowly with continuous agitation. The tubes were refrigerated at 4°C overnight before being spun at 1000 r.p.m. for ten minutes. Again the supernatant was removed and replaced with fresh fixative. After spinning the tubes at 1000 r.p.m. for another ten minutes, the supernatant was removed and replaced once more. For the last time the tubes were spun at 1000 r.p.m. for ten minutes. After removing the supernatant, the pellet was gently resuspended in 0.5 ml. of fixative. Using a pasteur pipette, two to three drops of this suspension were dropped at a height of approximately 30 cm. onto a cold wet slide. After allowing the excess liquid to drain, the slide was dried on a slide warmer. One slide for each culture was made and examined, unstained, under a light microscope using a 10X objective and the condenser lowered. The quality of the slide was judged on the amount of visible cytoplasm and the spreading of the cells and their chromosomes. If the slide was satisfactory, three more were made for each culture (ie. sixteen slides for each blood sample).

(f) Principles

The blood culture used in this study is an example of a short term in vitro culture of peripheral blood. The cells are put in medium only long enough to accumulate a sufficient number of mitoses needed for analysis. The cells are grown in medium that contains the necessary antibiotics and nutrients (ie. sugars, salts, amino acids, and vitamins) for cell growth and division (Zackai and Mellman, 1974).

As part of the culture, the mitogen phytohemagglutinin (PHA) is also added. Its function is to stimulate the lymphocytes to divide (Nowell, 1960). Within the first twenty-four hours of incubation the cells will show a significant increase in their synthesis of RNA. By forty-eight hours the nuclei will enlarge and DNA synthesis will have begun. The peak of mitotic activity and therefore the optimum time to harvest the culture will be

reached at seventy-two hours when approximately forty-five percent of the cells will be in S phase (eg. Mackinney et al., 1962).

Near the end of the incubation period, colcemid is added to arrest the cells at metaphase (Tjio and Levan, 1956; Ford and Hamerton, 1956). The extent to which the chromosomes contract will depend on the amount of colcemid and on the amount of time the cells are exposed to it (Zackai and Mellman, 1974).

In arresting the cells at metaphase the colcemid has prevented the formation of the spindle apparatus. Consequently the chromosomes are no longer attached to any binding force within the cell. Once the cells are suspended in hypotonic solution (Hsu, 1952) the resulting concentration gradient will cause water to rush into them and force their cell membranes to stretch far beyond their normal size. This sudden increase in cell volume will allow the chromosomes to spread out.

As part of the harvesting procedure, the hypotonic is eventually replaced with fixative (eg. Saksela and Moorhead, 1962). This mixture of acetic acid and methanol acts to (1) preserve the cells and their contents while at the same time stopping all cellular activity and (2) remove the concentration gradient formed by the hypotonic solution. The time at which the hypotonic solution is replaced is important. If it is replaced early, the chromosomes within each cell will have very little room to spread out from one another once the cells are dropped on to a slide. However if the hypotonic solution is replaced late, the cells will burst.

When the harvesting procedure is finished, the cells are dropped on to a wet slide. As the fixative hits the water the surface tension is temporarily broken forcing the cells to spread out. As the fixative evaporates the cells begin to flatten and, in doing so, force the chromosomes to spread out into a single layer. Creating a single layer of nonoverlapping chromosomes depends on a number of factors including (1) the height at which the cells are dropped on to a slide and (2) the rate at which the fixative evaporates on the slide. The latter will depend upon the atmospheric humidity and the temperature of the slide. For

example, in a dry climate the rate of fixative evaporation can be reduced by using a cold slide and also creating a slight increase in humidity above the slide by gently blowing on it.

5. Silver Staining of the Nucleolar Organizing Region (NOR).

The protocol used for the silver staining of NORs was the same as that used by the University of Alberta Hospitals' Cytogenetics Laboratory (Verma and Babu, 1984).

(a) Materials

1. prepared slides (aged at least one week)
2. 60°C waterbath
3. coverslips (No. 1)
4. disposable petri dishes
5. pasteur and graduated pipettes
6. coplin jars
7. 30 ml. bottle with screw on cap
8. photomicroscope

(b) Solutions and reagents

1. gelatin
2. distilled water
3. formic acid
4. silver nitrate
5. Giemsa stain
6. phosphate buffer (one phosphate buffer tablet (pH 6.8) per 1000 ml. of distilled water)

(c) Stock solutions

1. Solution A

After dissolving 2.0 gm. of gelatin in 100 ml. of distilled water, 1.0 ml. of formic acid was added. The mixture was stirred continuously for ten minutes and then stored at room temperature for a maximum of two weeks.

2. Solution B

In a 30 ml. bottle 4.0 gm. of silver nitrate was dissolved in 8.0 ml. of distilled water. The solution could be stored indefinitely in a light-tight container.

(d) Method

Using separate pasteur pipettes, two drops of solution A and four drops of solution B were placed on a prepared slide. Using a third pasteur pipette, the solutions were mixed. After covering the slide with a coverslip, it was placed in a petri dish and allowed to float in a 60°C waterbath. The slide was removed and rinsed with distilled water only after it had turned golden-brown.

To visualize the chromosomes, the slide was submerged in the Giemsa staining solution for fifteen seconds and then rinsed in distilled water. The slide was examined under a light microscope using the 100X objective lense. The silver staining procedure was repeated in cases were the NORs could not be visualized.

6. Identifying Double Nucleolar Organizing Regions (dNORs).

The NOR heteromorphisms were assessed under a light microscope using a 100X objective lense. For each subject approximately two slides were silver stained and examined for the presence of a dNOR on any of the acrocentric chromosomes. Slides of

poor quality (ie. the staining intensity was either too faint or too dark for a proper analysis) were discarded and replaced.

Since the purpose of this project was to assess the validity of the results of Jackson-Cook et al. (1985) their dNOR classification scheme was used in this study. Therefore a silver stain configuration was classified as a dNOR if it consisted of (1) two spots of silver stain per chromatid with a space between them or (2) two spots of silver stain per chromatid not separated by a clear area, but demonstrating a constriction that would suggest two areas of silver stain close together. In an acrocentric chromosome at metaphase one of the two spots of precipitated silver on each chromatid had to be proximal to the centromere while the other spot was distal to the centromere.

Jackson-Cook et al. (1985) did not specify the minimum percentage of cells that had to express a dNOR in order for the subject to be classified as a dNOR(+) carrier. In this study individuals were classified as dNOR(+) carriers if approximately ten percent of their cells expressed a dNOR. The best three examples of a dNOR variant chromosome were photographed.

7. Photography

The protocol used for photography was the same as that used by the University of Alberta Hospitals' Cytogenetics Laboratory.

(a) Film development

(i) Materials

1. Kodak Technical Pan Film 2415
2. developing tanks
3. film apron

4. beakers
5. measuring cylinder

(ii) Solutions

1. HC110 stock solution (Kodak)
2. acetic acid
3. water
4. Kodak Ektaflo fixer
5. hypo-clearing agent (Kodak)
6. Photo-flo (Kodak)

(iii) Working solutions (for the development of one roll of film)

1. Developer (50 ml. of HC110 stock solution added to 350 ml. of water)
2. Stop solution (400 ml. of water mixed with approximately 10 ml. of acetic acid)
3. Fixative (100 ml. of Ektaflo fixer mixed with 300 ml. of water)
4. Hypo-clearing agent (100 ml. of the stock solution mixed with 400 ml. of water)
5. Photo-flo (2 ml. of the stock solution added to 500 ml. of water)

(iv) Processing

The film was removed from its magazine in total darkness and rolled on to a film apron to be placed in a light-tight developing tank. With occasional agitation, the film was submerged in the developer for six minutes, followed by the stop solution (thirty seconds), the fixative (ten minutes), and finally the hypo-clearing agent (two minutes). After washing the film in water for ten minutes, it was rinsed in the photo-flo solution for thirty seconds and hung up to dry.

(b) Printing

(i) Materials

1. paper - Kodabromide F , single weight
2. film enlarger
3. print washer
4. print dryer
5. developing trays
6. measuring cylinder
7. safelights

(ii) Solutions

1. Dektol developer (Kodak)
2. water
3. acetic acid
4. hypo-clearing agent (Kodak)
5. Kodak Ektaflo fixer

(iii) Working solutions

1. Developer (500 ml. of Dektol added to 1000 ml. of water)
2. Stop bath (approximately 10 ml. of acetic acid added to 2000 ml. of water)
3. Fixative (300 ml. of Ektaflo fixer added to 1700 ml. of water)
4. Hypo-clearing agent (400 ml. stock solution added to 1600 ml. of water)

(iv) Processing

Under the safelights, the negative was placed in the enlarger and exposed on to a sheet of photo-paper for the required length of time (approximately fifteen seconds). To develop the exposed paper, it was first submerged in the developer for two minutes, followed by the stop solution (thirty seconds), the fixative (ten minutes), and finally the hypo-clearing agent (two minutes). After washing the paper in water for ten minutes, it was placed in the print dryer.

III. Results and Discussion

1. dNOR Configurations

Figures 6 to 22 represent all of the dNOR(+) carriers in the control group. Figures 23 to 30 represent all of the dNOR(+) carriers in test group A. Each figure consists of three partial karyotypes (i to iii) for one individual. In each partial karyotype, from left to right, is an example of a C-group chromosome, a D-group chromosome with a normal silver stained NOR on each chromatid, a G-group chromosome with a normal silver stained NOR on each chromatid, and either a D or G-group chromosome with a silver stained dNOR on each chromatid. The first three chromosomes in each partial karyotype were included to demonstrate the difference in appearance between (1) a dNOR and a normal NOR, (2) a D and G-group chromosome, and (3) an unstained and silver stained chromosome. Using Figure 6i as an example, like all nonacrocentric chromosomes the C-group chromosome does not have an NOR on its short arm (Ohno et al., 1961; Ferguson-Smith and Handmaker, 1961). Consequently it lacks the NOR-associated proteins that can react with the silver stain to yield the characteristic black spots of precipitated silver (eg. Howell et al., 1975). The D and G-group chromosomes in this figure can be distinguished from each other by the size of their long arms. The D-group chromosome has a long arm that is about the same length as that of the C-group chromosome. However the G-group chromosome has a long arm several times smaller than this. As acrocentric chromosomes in metaphase, both the D and G-group chromosome in this figure have an NOR on each chromatid which, when silver stained, appeared as a pair of black spots of precipitated silver. The fourth chromosome in Figure 6i is a D-group chromosome with a dNOR on the short arm of each chromatid (ie. two NORs per chromatid - one that is proximal to the centromere and one that is distal to it). Consequently, when this chromosome was silver stained four black spots of precipitated silver appeared.

FIGURES 6, 7, and 8. In each figure there are three silver (NOR) stained partial karyotypes of the control subject. In each partial karyotype, from left to right, is an example of a C-group chromosome, a D-group chromosome with a silver stained NOR, a G-group chromosome with a silver stained NOR, and a D-group chromosome with a silver stained dNOR.

i ii iii

FIGURE 6. Control subject No. 17

i ii iii

FIGURE 7. Control subject No. 46

i ii iii

FIGURE 8. Control subject No. 20

FIGURES 9, 10, and 11. In each figure there are three silver (NOR) stained partial karyotypes of the control subject. In each partial karyotype, from left to right, is an example of a C-group chromosome, a D-group chromosome with a silver stained NOR, a G-group chromosome with a silver stained NOR, and a D-group chromosome with a silver stained dNOR.

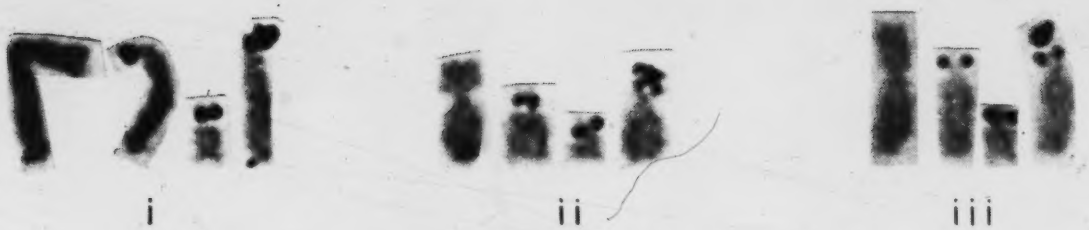

FIGURE 9. Control subject No. 49

FIGURE 10. Control subject No. 50

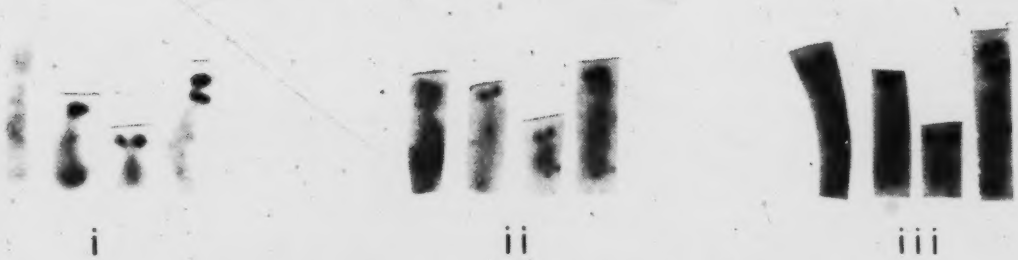

FIGURE 11. Control subject No. 98

FIGURES 12, 13, and 14. In each figure there are three silver (NOR) stained partial karyotypes of the control subject. In each partial karyotype, from left to right, is an example of a C-group chromosome, a D-group chromosome with a silver stained NOR, a G-group chromosome with a silver stained NOR, and a D-group chromosome with a silver stained dNOR.

i ii iii

FIGURE 12. Control subject No. 53

i ii iii

FIGURE 13. Control subject No. 4

i ii iii

FIGURE 14. Control subject No. 83

FIGURES 15, 16, and 17. In each figure there are three silver (NOR) stained partial karyotypes of the control subject. In each partial karyotype, from left to right, is an example of a C-group chromosome, a D-group chromosome with a silver stained NOR, a G-group chromosome with a silver stained NOR, and a D-group chromosome with a silver stained dNOR.

FIGURE 15. Control subject No. 10

FIGURE 16. Control subject No. 7

FIGURE 17. Control subject No. 75

FIGURES 18, 19, and 20. In each figure there are three silver (NOR) stained partial karyotypes of the control subject. In each partial karyotype, from left to right, is an example of a C-group chromosome, a D-group chromosome with a silver stained NOR, a G-group chromosome with a silver stained NOR, and a G-group chromosome with a silver stained dNOR.

i ii iii

FIGURE 18. Control subject No. 27

i ii iii

FIGURE 19. Control subject No. 31

i ii iii

FIGURE 20. Control subject No. 45

FIGURES 21 and 22. In each figure there are three silver (NOR) stained partial karyotypes of the control subject. In each partial karyotype, from left to right, is an example of a C-group chromosome, a D-group chromosome with a silver stained NOR, a G-group chromosome with a silver stained dNOR.

FIGURE 21. Control subject No. 1

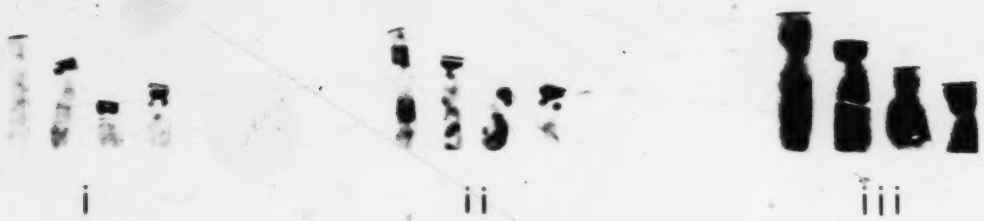

FIGURE 22. Control subject No. 74

FIGURES 23, 24, and 25. In each figure there are three silver (NOR) stained partial karyotypes of the test subject. In each partial karyotype, from left to right, is an example of a C-group chromosome, a D-group chromosome with a silver stained NOR, a G-group chromosome with a silver stained NOR, and a D-group chromosome with a silver stained dNOR.

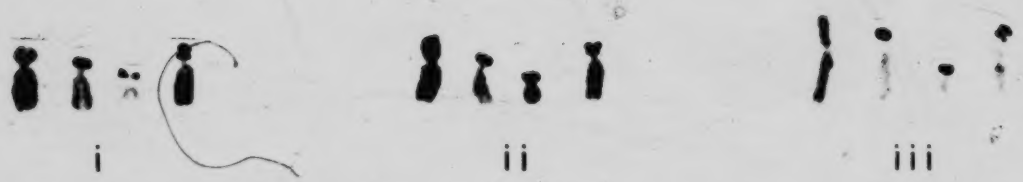

FIGURE 23. Male parent in couple No. 11 of test group A

FIGURE 24. Female parent in couple No. 11 of test group A

FIGURE 25. Male parent in couple No. 25 of test group A

FIGURES 26, 27, and 28. In each figure there are three silver (NOR) stained partial karyotypes of the test subject. In each partial karyotype, from left to right, is an example of a C-group chromosome, a D-group chromosome with a silver stained NOR, a G-group chromosome with a silver stained NOR, and a D-group chromosome with a silver stained dNOR.

FIGURE 26. Male parent in couple No. 5 of test group A

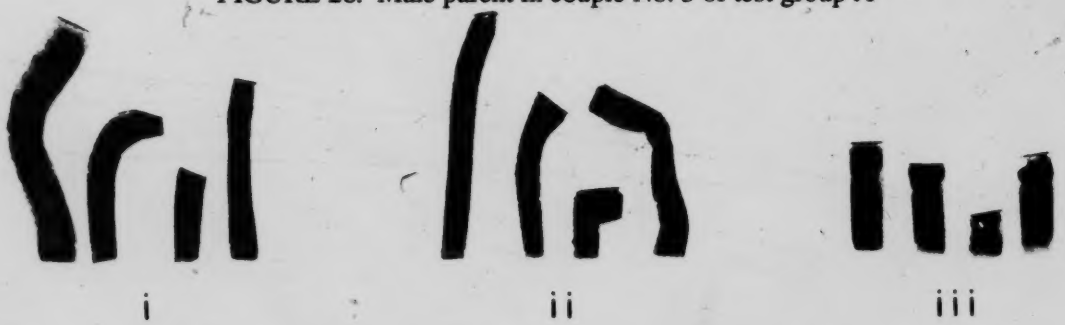

FIGURE 27. Male parent in couple No. 6 of test group A

FIGURE 28. Male parent in couple No. 18 of test group A

FIGURES 29 and 30. In each figure there are three silver (NOR) stained partial karyotypes of the test subject. In each partial karyotype, from left to right, is an example of a C-group chromosome, a D-group chromosome with a silver stained NOR, a G-group chromosome with a silver stained NOR, and a G-group chromosome with a silver stained dNOR.

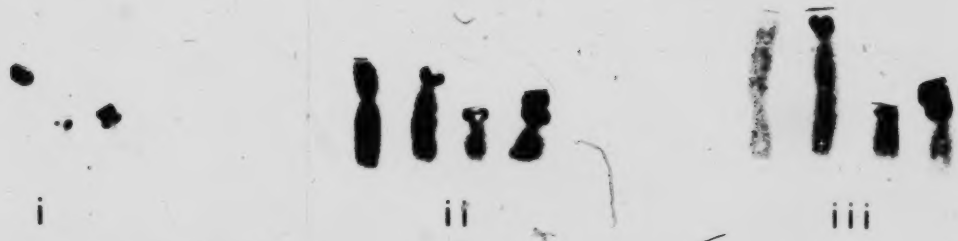

FIGURE 29. Female parent in couple No. 22 of test group A

FIGURE 30. Female parent in couple No. 14 of test group A

In many of the examples in Figures 6 to 30 of an acrocentric chromosome at metaphase with a normal NOR on each chromatid, silver staining had made the two NORs appear as one region of precipitated silver (eg. Figure 6iii - third chromosome). Similarly, in many of the examples of an acrocentric chromosome at metaphase with a dNOR on each chromatid, silver staining had made the two NORs proximal to the centromere (one on each chromatid) appear as one staining region and the two NORs distal to the centromere (one on each chromatid) appear as a second staining region (eg. Figure 6iii - fourth chromosome). These silver stain configurations were probably the result of an excess of precipitated silver on each NOR such that, when two NORs on different chromatids are located at the same position along the chromosome, their two silver staining regions intersect each other.

The fourth chromosome in Figure 9i is an example of two dNORs which when silver stained appeared together as one region of precipitated silver with a constriction. The amount of precipitated silver on each of the four NORs (two NORs per chromatid) was at a level that forced all four staining regions to intersect each other.

For the fourth chromosome in Figure 9iii, silver staining had made the two dNORs appear together as a tripartite structure. Only the NORs distal to the centromere had enough precipitated silver to intersect each other.

These examples of acrocentric chromosomes at metaphase with silver stained NORs (one NOR per chromatid) and silver stained dNORs (two NORs per chromatid), illustrate the variability in silver stain configurations that were observed in this study. The implications of this variability, with respect to dNOR classification, will be discussed later.

2. The Distribution of dNORs in the Control Group.

The distribution of dNOR(+) carriers in the control group (ie. individuals with no chromosomal abnormality or with a chromosomal abnormality other than trisomy) is

presented in Table 3. Table 3 presents each subject's sex, age at the time the blood sample was taken, clinical indications (ie. the reason the blood sample was taken for chromosome analysis at the University of Alberta Hospitals' Cytogenetics Laboratory), karyotype, dNOR classification (if the subject was a dNOR(+) carrier, the type of chromosome carrying the NOR variant was indicated), and the percentage of cells expressing the dNOR.

For an easier interpretation of the incidence of dNOR(+) carriers in the control group, the pertinent data in Table 3 were summarized in Table 4. Each subject was categorized by age and dNOR classification and subcategorized by sex. The total number of subjects in each category/subcategory and the percentage of dNOR(+) carriers in each age group were included in the table.

Under the heading "Clinical Indications", Table 3 presents the reason each subject had a blood sample analysed by the cytogenetics laboratory. In many cases the subject had a number of physical and/or mental defects which were suggestive of a syndrome such as Down Syndrome, Turner Syndrome, Klinefelter Syndrome, Fragile X Syndrome and Prader Willi Syndrome. A chromosomal abnormality was also suspected in several individuals with only one defect such as delayed or premature physical development, malformed or ambiguous genitalia, or mental retardation. Infertility, recurrent abortions, or a family history of a genetic disorder were the circumstances which prompted the chromosome analysis of blood taken from a number of other subjects in the control group. This test was to determine if these individuals were translocation carriers.

The karyotypes of all but two people in the control group were normal (ie. 46,XX or 46,XY). The exceptions were a translocation carrier and a carrier of an inversion. Both individuals were infertile.

Table 3 shows that the control group consisted of individuals varying in age from a newborn to a seventy-three year old male (most of the subjects were under the age of thirty-five). Since the subjects were chosen at random with respect to age (See Materials and

TABLE 3

Distribution of Double NORs in the Control Group
(100 individuals with no chromosomal abnormality or with an abnormality other than trisomy)

Subject	Male/Female	Age	Clinical Indications	Karyotype	dNOR(-)/dNOR(+)	% of cells dNOR(+)
1.	Female	25	premature menopause	46,X, inv dup(X)	+ (G-group)	9.0
2.	Male	5	X-linked Muscular Dystrophy	46,XY	-	0.0
3.	Male	10	Fragile X	46,XY	-	0.0
4.	Female	22	Retino-blastoma	46,XX	+ (D-group)	7.0
5.	Female	N	Trisomy 21	46,XX	-	0.0
6.	Male	11	relative 46,XX inv(9)	46,XY	-	0.0
7.	Female	5	enlarged clitoris	46,XX	+ (D-group)	11.0
8.	Female	25	recurrent aborter	46,XX	-	0.0
9.	Male	4	left undescended testes	46,XY	-	0.0
10.	Female	30	recurrent aborter	46,XX t(11;22)	+ (D-group)	8.0
11.	Male	28	normal	46,XY	-	0.0
12	Male	13	Fragile X	46,XY	-	0.0
13.	Male	73	Myelodys-plastic syndrome	46,XY	-	0.0
14.	Male	26	mental-retardation tall stature, aggressive behaviour	46,XY	-	0.0

15.	Female	28	recurrent aborter	46,XX	-	0.0
16.	Female	20	Fragile X	46,XX	-	0.0
17.	Male	38	wife is a recurrent aborter 46X/46,XX	46,XY	+ (D-group)	17.0
18.	Female	21	Rokitansky Syndrome	46,XX	-	0.0
19.	Male	7	Fragile X	46,XY	-	0.0
20.	Male	15	VSD, Robins-Tread Syndrome	46,XY	+ (D-group)	16.0
21.	Female	30	relative is a translocation carrier t(14;21)	46,XX	-	0.0
22.	Female	30	X variant	46,XX	-	0.0
23.	Female	22	relative is a translocation carrier t(11;21)	46,XX	-	0.0
24.	Male	9	Fragile X	46,XY	-	0.0
25.	Male	32	Klinefelter Syndrome	46,XY	-	0.0
26.	Male	4	hypospadias	46,XY	-	0.0
27.	Female	13	short stature	46,XX	+ (G-group)	21.0
28.	Female	33	relative is a translocation carrier t(13;14)	46,XX	-	0.0
29.	Male	20	overweight, striae	46,XY	-	0.0
30.	Female	15	Turner Syndrome	46,XX	-	0.0

31.	Female	32	relative is a translocation carrier t(1;13)	46,XX	+ (G-group)	8.0
32.	Female	5	ambiguous genitalia	46,XX	-	0.0
33.	Male	23	relative is a translocation carrier t(14;17)	46,XY	-	0.0
34.	Male	6	Fragile X	45,XY	-	0.0
35.	Female	6	growth delay	46,XX	-	0.0
36.	Female	1	short stature	46,XX	-	0.0
37.	Male	18	Fragile X	46,XY	-	0.0
38.	Male	11	Fragile X	46,XY	-	0.0
39.	Male	31	infertility	46,XY	-	0.0
40.	Female	2	Prader Willi Syndrome	46,XX	-	0.0
41.	Female	24	X variant	46,XX	-	0.0
42.	Female	34	child with dysmorphic features	46,XX	-	0.0
43.	Female	N	Fragile X	46,XX	-	0.0
44.	Female	5	Prader Willi	46,XX	-	0.0
45.	Female	8	multiple malformations	46,XX	+ (G-group)	16.0
46.	Male	19	Klinefelter Syndrome	46,XY	+ (D-group)	17.0
47.	Male	24	mental retardation	46,XY	-	0.0
48.	Male	44	Kelly-Harvey ring 18 mosaic	46,XY	-	0.0

49.	Female	33	recurrent aborter	46,XX	+ (D-group)	14.0
50.	Female	24	child 18p+	46,XX	+ (D-group)	9.0
51.	Female	32	infertility	46,XX	-	0.0
52.	Female	35	relative is a translocation carrier t(13;14)	46,XX	-	0.0
53.	Male	13	precocious puberty, mental-retardation	46,XY	+ (D-group)	10.0
54.	Male	27	wife is a recurrent aborter	46,XY	-	0.0
55.	Male	1	Bowen-Conradi Syndrome	46,XY	-	0.0
56.	Male	29	normal	46,XY	-	0.0
57.	Male	21	Down Syndrome	46,XY	-	0.0
58.	Female	33	relative is a translocation carrier t(14;21)	46,XX	-	0.0
59.	Female	21	Fragile X	46,XX	-	0.0
60.	Male	14	growth delay	46,XY	-	0.0
61.	Female	32	relative is a translocation carrier t(14;21)	46,XX	-	0.0
62.	Female	20	relative is a translocation carrier t(18;G)	46,XX	-	0.0

63.	Male	28	relative is a translocation carrier t(13;14)	46,XY	-	0.0
64.	Female	1	motor delay, dysmorhpic features	46,XX	-	0.0
65.	Male	1	Klinefelter Syndrome	46,XY	-	0.0
66.	Female	29	child with dysmorphic features	46,XX	-	0.0
67.	Female	12	Turner Syndrome	46,XX	-	0.0
68.	Female	38	relative is a translocation carrier t(14;21)	46,XX	-	0.0
69.	Male	7	develop-mental delay	46,XY	-	0.0
70.	Female	22	unusual pelvic organs, streak ovaries	46,XX	-	0.0
71.	Female	23	recurrent aborter	46,XX	-	0.0
72.	Female	7	short stature	46,XX	-	0.0
73.	Female	26	Turner Syndrome	46,XX	-	0.0
74.	Male	3	dysmorphic features	46,XY	+ (G-group)	23.0
75.	Male	N	born with single ear	46,XY	+ (D-group)	19.0
76.	Male	29	relative is a translocation carrier t(9;22)	46,XY	-	0.0

77.	Male	1	Fragile X	46,XY	-	0.0
78.	Female	24	child born with no left hand	46,XX	-	0.0
79.	Female	1	inguinal hernia	46,XX	-	0.0
80.	Male	35	congenital anomalies	46,XY	-	0.0
81.	Male	38	infertility	46,XY	-	0.0
82.	Female	29	relative is a translocation carrier t(14;21)	46,XX	-	0.0
83.	Female	31	recurrent aborter	46,XX	+ (D-group)	14.0
84.	Male	27	relative is a translocation carrier t(13;14)	46,XY	-	0.0
85.	Male	8	small testis	46,XY	-	0.0
86.	Male	4	pyloric-stenosis, failure to thrive	46,XY	-	0.0
87.	Male	29	relative is a translocation carrier t(14;21)	46,XY	-	0.0
88.	Male	11	Fragile X	46,XY	-	0.0
89.	Female	38	child has delayed development	46,XX	-	0.0
90.	Male	29	wife is a recurrent aborter	46,XY	-	0.0

91.	Male	17	tall, over-weight, borderline intelligence	46,XY	-	0.0
92.	Male	1	Prader Willi Syndrome	46,XY	-	0.0
93.	Female	9	small stature	46,XX	-	0.0
94.	Female	38	infertility	46,XX	-	0.0
95.	Female	2	speech delay	46,XX	-	0.0
96.	Female	32	Prader Willi Syndrome	46,XX	-	0.0
97.	Female	13	relative is a translocation carrier t(13;14)	46,XX	-	0.0
98.	Female	28	recurrent aborter	46,XX	+ (D-group)	12.0
99.	Female	30	secondary amenorrhea	46,XX	-	0.0
100.	Male	34	mental retardation in family	46,XY	-	0.0

N = newborn

TABLE 4

Age Distribution of the 100 Individuals in the Control Group

Age	dNOR(-) Male	dNOR(-) Female	dNOR(+) Male	dNOR(+) Female	Total	%*
0 - 4	8	7	2	0	17	11.8
5 - 9	6	4	0	2	12	16.7
10 - 14	5	2	1	1	9	22.2
15 - 19	2	2	2	0	6	33.3
20 - 24	3	8	0	2	13	15.4
25 - 29	11	6	0	2	19	10.5
30 - 34	3	9	0	4	16	25.0
≥ 35	3	4	1	0	8	12.5
Sub-total	41	42	6	11		
Total	83		17		100	17.0

* The percentage of individuals in each age group with a dNOR.

Methods) their distribution among the various age groups (See Table 4) was fairly representative of the kinds of people whose blood samples were sent to the University of Alberta Hospitals' Cytogenetics Laboratory. The total number of people in each age group show that the majority of blood samples were either from adults, mainly females in their childbearing years, or from newborns and young children (an even distribution of males and females).

From Table 4, the distribution of dNOR(+) carrier males and females within the control group follows the same distribution pattern with respect to age as the dNOR(-) individuals (ie. a fairly even number of males and females within each age group from birth to nineteen years (and beyond thirty-five years) and an excess of females within the ages of twenty to thirty-four). In total there was no significant difference in the number of dNOR(+) males and females. (ie. 6/47 males and 11/53 females, $\chi^2 = 0.95$, $p > .05$).

The percentage of individuals in each age group that were dNOR(+) carriers was calculated and presented in Table 4. Unfortunately the number of individuals in each age group was too low for the percentages to be considered significant. Also calculated was the percentage of individuals in the entire control group that were dNOR(+) carriers. At seventeen percent, one may conclude that this was a common NOR variant in the control group.

In reality the frequency of dNOR(+) carriers in the control group might have been slightly higher or lower than seventeen percent. It might have been lower because a dNOR(+) carrier's inclusion into the control group was dependent on his, or his family's, recollection of family history regarding evidence of trisomic pregnancies. The value might have been higher if some of the supposed dNOR(-) individuals were actually dNOR(+) carriers. Reasons for a possible false negative identification will be discussed later.

For each of the dNOR(+) carriers in the control group a silver stained dNOR was not seen in every cell. As shown in Table 3, the observed percentage of cells in which the dNOR was expressed ranged from seven to twenty-three percent.

3. The Distribution of dNORs in Test Group A.

The distribution of dNOR(+) carriers in test group A (ie. couples with one child with Down Syndrome) is presented in Table 5. Also included in this table is the age of the female parent in each couple and the percentage of cells that expressed a dNOR in each dNOR(+) carrier.

The maternal ages ranged from twenty-one to thirty-five years.

The observed percentage of cells in which a dNOR was expressed ranged from ten to twenty-four percent.

In six of the twenty-five couples (ie couples 5, 6, 14, 18, 22, and 25) one parent was a dNOR(+) carrier. In a seventh couple (ie. couple 11) both parents were dNOR(+) carriers. These results show that in test group A twenty eight percent (7/25) of the couples had a dNOR in at least one parent. Examining all of the parents in test group A individually, sixteen percent (8/50) of the people were dNOR(+) carriers.

Comparing the results of the control group with those of test group A, there does not appear to be a significant difference in their incidence of dNOR(+) carriers ($\chi^2 = .03$, $p > .05$).

4. The distribution of dNORs in Test Group B.

Test group B was made up of five families. Each had more than one trisomic child. Only the parents of the affected children were examined for the presence of dNORs.

In family No.1 (See Figure 31), in which two siblings were trisomic for chromosome 21, neither parent carried a dNOR on any of their acrocentric chromosomes.

In family No.2. (See Figure 32), a brother (II-1) and sister (II-3) were the parents of a child with Trisomy 21 (III-1) and a child with Trisomy 13 (III-3) respectively. Only

TABLE 5

Distribution of Double NORs in Test Group A
(25 couples with a Down Syndrome child)

Couple	Maternal age	dNOR(-)/dNOR(+)		% of cells dNOR(+)	
		Male	Female	Male	Female
1.	21	-	-	/	/
2.	21	-	-	/	/
3.	22	-	-	/	/
4.	23	-	-	/	/
5.	23	+ (D-group)	-	24.0	/
6.	23	+ (D-group)	-	10.0	/
7.	24	-	-	/	/
8.	25	-	-	/	/
9.	26	-	-	/	/
10.	26	-	-	/	/
11.	26	+ (D-group)	+ (D-group)	10.0	15.0
12.	27	-	-	/	/
13.	27	-	-	/	/
14.	27	-	+ (G-group)	/	12.0
15.	30	-	-	/	/
16.	30	-	-	/	/
17.	31	-	-	/	/
18.	31	+ (D-group)	-	10.0	/
19.	32	-	-	/	/
20.	33	-	-	/	/
21.	34	-	-	/	/

22.	34	-	+ (G-group)	/	11.0
23.	35	-	-	/	/
24.	35	-	-	/	/
25.	35	+ (D-group)	-	14.0	/

Total number of dNOR(+) individuals in test group A: 8

Total number of dNOR(-) individuals in test group A: 42

Percentage of individuals in test group A that are dNOR(+) carriers: 16.0

FIGURE 31. Pedigree for family No. 1 of test group B

In this family the affected children (II-1 and II-2) were siblings. Neither parent was a dNOR(+) carrier

FIGURE 32. Pedigree for family No. 2 of test group B

In this family the affected children (III-1 and III-3) were first cousins. Both parents of III-1 were dNOR(+) carriers. Only the father of III-3 was a dNOR(+) carrier.

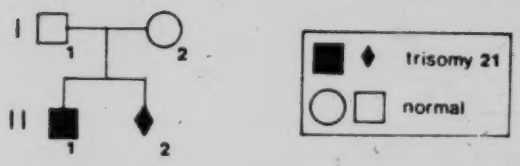

FIGURE 31. Pedigree for family No. 1 of test group B

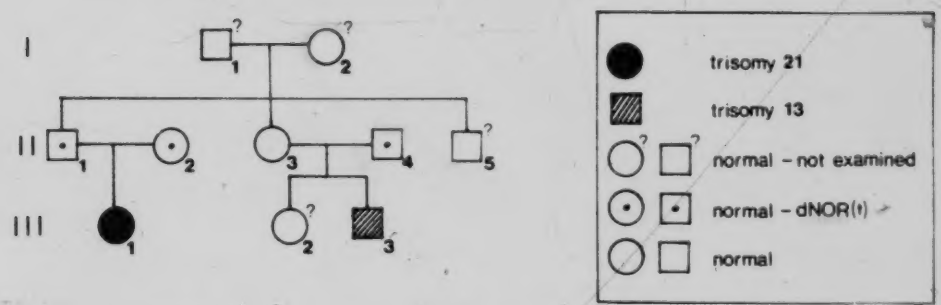

FIGURE 32. Pedigree for family No. 2 of test group B

II-1 carried a dNOR (See Figure 33). The dNOR was found on a D-group chromosome in seventeen percent of the cells examined. His wife (II-2) and brother-in-law (II-4) were also dNOR(+) carriers (See Figure 34 (II-2) and Figure 35 (II-4)). Their dNORs were found on G-group chromosomes. In II-2 the dNOR was expressed in eight percent of the cells examined and in II-4 the dNOR was expressed in ten percent of the cells examined. None of the dNOR(+) carriers were related.

In family No.3. (See Figure 36), a brother (II-1) and sister (II-3), were each parents of a child with Trisomy 21 (III-1 and III-2 respectively). In this situation both siblings had a dNOR on a G-group chromosome (See Figure 38 (II-1) and Figure 37 (II-3)). The dNOR was expressed in nine percent of the cells examined in II-1 and in ten percent of the cells examined in II-3.

In family No.4. (See Figure 39), I-3 and his nephew (II-2) were each parents of a child with Trisomy 21 (II-4 and III-2 respectively). Only II-2 was a dNOR(+) carrier (See Figure 40). The dNOR was located on a D-group chomosome. It was expressed in fourteen percent of the cells examined. The wife of I-3 (I-4), who was not related to II-2, was also a dNOR(+) carrier (See Figure 41). The dNOR was found on a G-group chromosome. It was expressed in ten percent of the cells examined.

In family No.5. (See Figure 42), one couple had two trisomic children. One child had Down Syndrome (II-1) and a second child was trisomic for the X chromosome (II-2). Neither parent was a dNOR(+) carrier. This family was included in test group B because of the report by Jones et al. (1987). It will be discussed later.

In three of the five families in test group B, each couple with an affected child had at least one parent that was a dNOR(+) carrier (ie. seven of the sixteen parents in this test group had a dNOR). Because the sample size of this group was very small these results may not be accurate.

FIGURES 33, 34, and 35. In each figure there are three silver (NOR) stained partial karyotypes of the test subject. In each partial karyotype, from left to right, is an example of a C-group chromosome, a D-group chromosome with a silver stained NOR, a G-group chromosome with silver stained NOR, and either a D-group chromosome (test subjects II-1 and II-2) or a G-group chromosome (test subject II-4) with a silver stained dNOR.

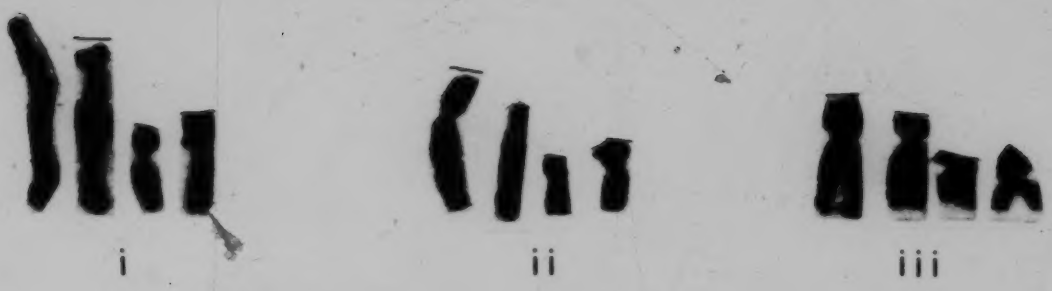

FIGURE 33. Parent II-1 in family No. 2 of test group B

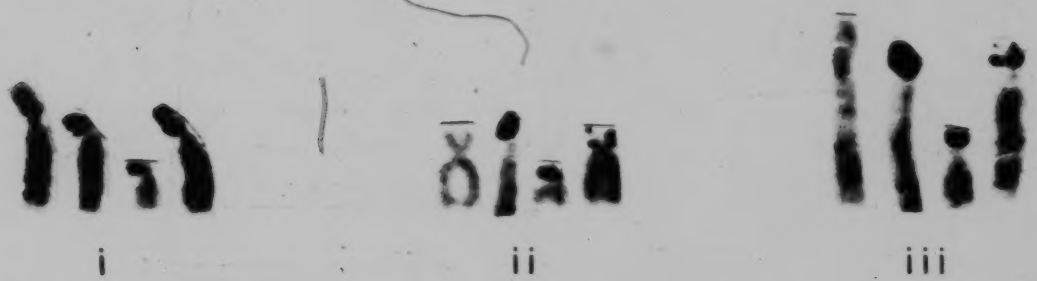

FIGURE 34. Parent II-2 in family No. 2 of test group B

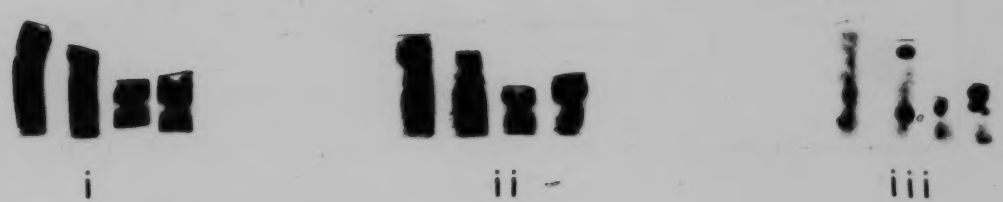

FIGURE 35. Parent II-4 in family No. 2 of test group B

FIGURE 36. Pedigree for family No. 3 of test group B

In this family the affected children (III-1 and III-2) were first cousins. The father of III-1 and the mother of III-2 were dNOR(+) carriers.

FIGURES 37 and 38. In each figure there are three silver (NOR) stained partial karyotypes of the test subject. In each partial karyotype, from left to right, is an example of a C-group chromosome, a D-group chromosome with a silver stained NOR, a G-group chromosome with a silver stained NOR, and a G-group chromosome with a silver stained dNOR.

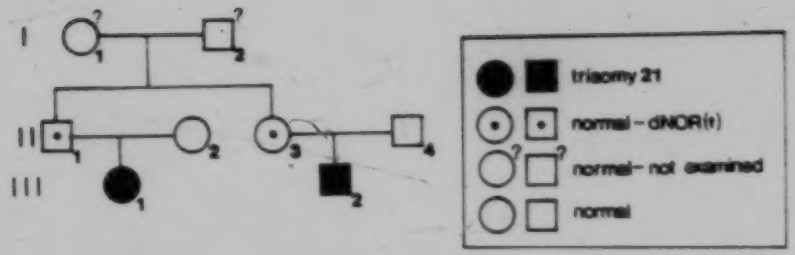

FIGURE 36. Pedigree for family No. 3 of test group B

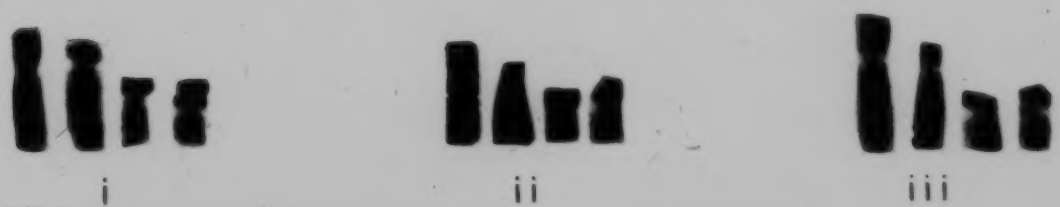

FIGURE 37. Parent II-3 in family No. 3 of test group B

FIGURE 38. Parent II-1 in family No. 3 of test group B

FIGURE 39. Pedigree for family No. 4 of test group B

In this family the affected children (II-4 and III-2) were second cousins. The mother of II-4 and the father of III-2 were dNOR(+) carriers.

FIGURES 40 and 41. In each figure there are three silver (NOR) stained partial karyotypes of the test subject. In each partial karyotype, from left to right, is an example of a C-group chromosome, a D-group chromosome with a silver stained NOR, a G-group chromosome with a silver stained NOR, and either a D-group chromosome (test subject II-2) or a G-group chromosome (test subject I-4) with a silver stained dNOR.

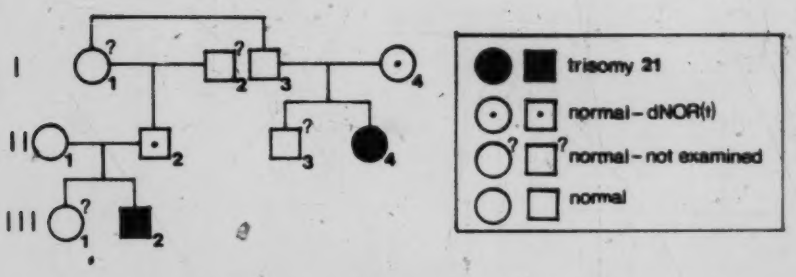

FIGURE 39. Pedigree for family No. 4 of test group B

FIGURE 40. Parent II-2 in family No. 4 of test group B

FIGURE 41. Parent I-4 in family No. 4 of test group B

FIGURE 42. Pedigree for family No. 5 of test group B

In this family the affected children (II-1 and II-2) were siblings. Neither parent was a dNOR(+) carrier.

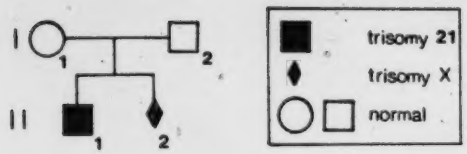

FIGURE 42. Pedigree for family No. 5 of test group B

Based only on the results of the control group and of test group A the dNOR does not appear to be a factor in nondisjunction. However, the limited results of test group B suggest that, at this time, it may be premature to make this conclusion.

5. A Comparison of Results Obtained by Different Research Groups.

Comparing the results of the present study with that of others (See Table 6), the first thing to note is the wide variation in data. Particularly noticeable within many of the studies is the lack of dNOR(+) carriers in both the test and control populations. There are a few explanations for this, not the least of which is a difference in opinion among the research groups as to which silver stain configurations should be classified as dNORs.

Most individuals (eg. Hassold, 1987; Hassold et al., 1987; Patil, 1987; Soukup, 1987) consider the dNOR classification scheme of Jackson-Cook et al. (1985) to be a liberal one. They would prefer to limit the definition of a silver stained dNOR to a structure consisting of two discrete bodies of precipitated silver per chromatid (eg. Soukup, 1987).

What disturbs most cytogeneticists is the inclusion by Jackson-Cook et al. (1985) of a dumbbell-shaped silver stain configuration as a dNOR. In theory a single region of silver stain demonstrating a constriction could be suggestive of two NORs close together (one proximal to the centromere and one distal to the centromere) but, in practice, differentiating between a dumbbell and an intensely stained single NOR is often confusing and may lead to an inaccurate estimate of dNOR frequency.

Rather than ignoring the dumbbell-shaped silver stain configuration altogether, Melnyk et al. (1987) used an approach similar to the one adopted in the present study. Although the authors used the dNOR classification scheme of Jackson-Cook et al. (1985), they placed a greater emphasis on finding dNORs with two discrete spots of silver-stain per chromatid rather than on the other dNOR configurations. An individual was considered a

TABLE 6

Incidence of Double NORs: A comparison of results obtained by different research groups.

Study	Test Population* number	%	Control Population number	%
Trunca (1986)**	0/8	0.0	/	/
Patil (1986)**	0/20	0.0	/	/
Warburton (1986)**	0/8	0.0	/	/
Hassold (1986)**	1/12	8.3	/	/
Hassold et al. (1987)	0/44	0.0	0/106	0.0
Wang and Uchida (1986)**	2/400	0.5	2/400	0.5
Soukup (1986)**	2/100	2.0	3/100	3.0
Wang (1986)**	3/198	1.5	2/200	1.0
Jones et al. (1987)	/	/	4/45	8.8
Jackson-Cook et al. (1985)	16/100	16.0	0/50	0.0
Melnyk et al. (1987)	13/64	20.3	9/97	9.3
Spinner et al. (1986)	6/48	12.5	6/38	15.8
Present study	8/50	16.0	17/100	17.0
Total	51/1052	4.8	43/1136	3.8

* The test populations consist of couples (both parents) with one Down Syndrome child.

** The data from these research groups was collected by T. Hassold in a phone survey (Hassold, 1987).

dNOR(+) carrier if, in examining twenty-five cells, they found at least four cells with two silver-stained bodies per chromatid and various other forms of the dNOR in the rest.

Spinner (1987) had developed a similar approach by stressing the importance of finding silver stain configurations in what she referred to as class No.1. This included all silver stain configurations with two discrete spots of silver stain per chromatid with a clear space between them. A class No.2 pattern consisted of two spots of silver stain per chromatid not separated by a clear area, but demonstrating a constriction suggesting two areas of silver stain close together. Class No.3 contained acrocentric chromosomes with a single large area of silver stain per chromatid approximately equal in size to the short arm of chromosome 18 in the same cell. Whereas, class No.4 consisted of all those acrocentrics with a single, small area of silver stain per chromatid or no silver stain.

A second explanation for the lack of agreement in data among several of the reports whose results are presented in Table 6 is that many of the dNOR(+) carriers may have been overlooked because of a low level of ribosomal gene activity (ie. the dNORs were only silver stained in a few cells). In the present study, a dNOR was identified in approximately ten to twenty percent of the cells examined in each carrier. In contrast, Jackson-Cook et al. (1985) observed a higher level at fifty to one hundred percent. If the same number of cells per subject were examined in the present study as were examined by Jackson-Cook et al. (1985) (ie. ten cells per subject), several dNOR(+) carriers probably would have been overlooked. Assuming that the same low level of ribosomal gene activity had occurred unnoticed in other studies, most of their dNOR(+) carriers would also have been overlooked.

A reason for the difference in ribosomal gene activity between the subjects of the present study and those of the study by Jackson-Cook et al. (1985) is not known. Perhaps it may be related to differences in methodology used. For example, de Capoa et al. (1985) discovered that ribosomal gene activity can be altered by environmental changes in the culture medium. In one of their experiments the authors were able to enhance gene activity

by increasing the amount of fetal calf serum (FCS) added to the culture from the standard five percent to ten and twenty percent (See Table 7).

A third reason for an under-representation of dNOR(+) carriers could be attributed to techniques in silver staining. There are several methods and some of these are time consuming and difficult (eg. Bloom and Goodpasture, 1976). A major problem is in standardizing the development time (ie. the amount of time that the prepared slide is exposed to the silver stain.). If this is done incorrectly, over and/or underdeveloped silver NORs (AgNORs) can result. While slides exhibiting the latter can be restained, slides with overdeveloped AgNORs must be discarded. Similar to the overdevelopment of a photograph, the overdevelopment of a dNOR can lead to a decrease in resolution of the two NORs on each chromatid. One might therefore overlook a dNOR because it could appear as a single silver-stained region.

As a consequence of differences in techniques and dNOR scoring, different groups of workers were not able to arrive at the same proportion of dNOR(+) carriers as Jackson-Cook et al. (1985). However, assuming that each research group produced an unbiased assessment of the frequency of dNORs in the control population and in the parents of children with Trisomy 21, one cannot deny the fact that unlike Jackson-Cook et al. (1985) they were unable to detect a significant difference in the frequency of dNORs between the test and control populations. The test and control populations for the combined results of the first eight studies listed in Table 6 gave almost identical dNOR frequencies (ie. 8/790(test) and 7/806 (control)). These observations agree with the results of the present study and with those of Spinner et al. (1986). While both studies were able to detect dNORs at the level of Jackson-Cook et al. (1985) (suggesting similar approaches in techniques and dNOR scoring), neither observed a significant excess of dNORs among the parents of Down Syndrome children over the control population. In the study by Spinner et al. (1986) dNORs were observed in six of thirty-eight controls (15.8 %) and in six of forty-eight parents of Down Syndrome children (12.5%). In the present study dNORs

TABLE 7

Distribution of silver stained NORs per cell in leucocyte cultures grown in 5% (A), 10% (B), and 20% (C) fetal calf serum.

Number of acrocentric chromosomes per cell with a silver stained NOR	Percentage of Cells		
	A*	B*	C*
1	-	-	-
2	-	-	-
3	-	-	-
4	2	-	-
5	11	4	1
6	26	16	8
7	40	39	30
8	21	39	59
9	-	2	2
10	-	-	-

* [A, $\bar{x} = 6.73$, $\bar{n} = 116$; B, $\bar{x} = 7.15$, $\bar{n} = 219$; C, $\bar{x} = 7.56$, $\bar{n} = 210$]
(Data from Figure 1 of de Capoa et al., 1985)

were observed in seventeen of one hundred controls (17.0%) and in eight of fifty parents of Down Syndrome children (16.0%).

In support of Jackson-Cook et al. (1985) are the studies by Melnyk et al. (1987) and Jones et al. (1987). The former used culturing, staining and dNOR scoring techniques similar to those of the present study. Still, they were able to obtain different results. Of sixty-four parents of Down Syndrome children, thirteen were dNOR(+) carriers (20.3%). Only nine of ninety-seven (9.3%) control subjects (newborns) were also dNOR(+) carriers.

Because of a possible association of the sex chromosomes with the acrocentric chromosomes during prophase I of meiosis (Solari and Tres, 1970), Jones et al. (1987) postulated that there might be a relationship between the presence of dNORs and the nondisjunction of sex chromosomes. To test this hypothesis, they compared the frequency of dNORs in Turner Syndrome patients (either 45,X or 45,X/46,XX) and normal volunteers. Surprisingly they found dNORs in fourteen of twenty-eight Turner Syndrome patients (50%). Only four of forty-five (8.8%) control subjects were also dNOR(+) carriers.

6. A Critical Evaluation of the Results and Conclusions Made by Jackson-Cook et al.(1985).

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IV. Conclusions

In the present study the incidence of dNOR(+) carriers in the control group and in test group A were almost identical. In the majority of comparable studies (See Table 6) the results were the same. Even differences in techniques and dNOR scoring, which prevented several groups of workers from detecting many dNOR(+) carriers, could not hide the fact that, in each, the dNOR frequencies in the test and control populations were not significantly different. These results suggest that the dNOR is not a factor in nondisjunction.

Perhaps the observation of Jackson-Cook et al. (1985) of a high incidence of dNORs among the parents of trisomic offspring was only a coincidental finding. Given the criticisms that have been made of the study, this may be true. Nevertheless, excluding the dNOR as a factor in nondisjunction may be premature given the results of test group B in the present study and the results of Melnyk et al. (1987) and Jones et al. (1987). Therefore, it is important that further studies be carried out.

If additional studies are pursued, the sample size of the test and control populations must be larger than in previous work to make the results significant.

For the control population, in addition to examining the frequency of dNORs in various age groups and in each sex, examining its frequency in various ethnic groups should also be considered. Present evidence indicates that there is little variation in the incidence of trisomy with respect to race and geographic location (eg. Hook, 1981). If a dNOR is a factor in nondisjunction, its frequency and effect should also be constant world wide

With the exception of two people, the control group in the present study consisted of individuals with normal karyotypes. However, based on the reason each blood sample was sent to the University of Alberta Hospitals' Cytogenetics Laboratory for chromosome

analysis, the incidence of dNORs found in this control group may not be representative of the general population. Therefore a second control group is needed. It should consist of individuals that (1) are phenotypically normal, (2) do not have a family history of a chromosomal disorder(s), and (3) upon chromosome analysis, show no chromosomal abnormality. The newborn population would be a good source of normal subjects because blood samples would be easy to obtain (ie. umbilical cord blood). Another possible source of normal subjects would be women undergoing an amniocentesis or chorion villi biopsy, provided the prenatal test(s) were done only because of the mother's advanced age. If the incidence of dNORs in the two control groups were found not to be significantly different from each other, then the conclusions made in the present study should be valid.

In addition to determining the frequency of dNORs in the parents of children with Trisomy 21, its frequency should also be determined in the parents of trisomies 13, 14, 15, and 22. Hassold et al. (1987) conducted a study which examined the frequency of dNORs among parents of spontaneous abortions trisomic for any of the acrocentric chromosomes. For comparison, they included a control group consisting of couples with chromosomally normal spontaneous abortions or with abortions having chromosome abnormalities presumably unrelated to NOR status. The authors were unable to detect dNORs in either group. While this was most likely due to difficulties in dNOR scoring, Hassold et al. (1987) considered a number of explanations. One which seems unlikely, but must be addressed in fairness to Jackson-Cook et al. (1985), is the possibility that dNORs are associated with the nondisjunction of only certain acrocentric chromosomes (eg. chromosome 21). If this is true, this effect would not have been detected in their study. Determining the frequency of dNORs in the parents of all acrocentric trisomies would resolve this issue.

Based on the results of de Capoa et al. (1985), ribosomal gene activity may be altered by environmental factors in the blood culture. Therefore in future studies to determine the incidence of dNORs in control and test populations it would be informative to

determine if the incidence increases with, for example increasing amounts of fetal calf serum (FCS) added to each blood culture. Perhaps in some individuals, at lower amounts of FCS the percentage of cells expressing a dNOR is too low for the NOR variant to be detected. It would also be informative to obtain from each subject two blood samples taken at different times. Perhaps physiological changes within the subject (eg. hormonal changes) may affect ribosomal gene activity and consequently the percentage of cells which express dNORs.

In the present study an attempt was made to examine the frequency and the distribution of dNORs in families with more than one acrocentric trisomy. Families of this type represent a very small portion of any population . Further work on this group would require a collaborative effort among several laboratories across the country in order to acquire an adequate sample size.

Finally, observing a statistically significant increase in the incidence of dNORs in cases of Turner Syndrome over controls, Jones et al. (1987) have suggested that dNORs may be a factor in the nondisjunction of the sex chromosomes. This study is worth repeating.

Before these and any other investigations are to be considered, there must be an agreement among cytogeneticists as to which silver stain configurations will be classified as dNORs. Until this is done, the current variation in results will continue and the controversy over whether the dNOR should be accepted as a factor in nondisjunction will never be resolved.

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APPENDIX 1

The Explanatory Letter Given to Subjects in Test Group A

Dear

We are writing to you in order to explain a research project in which we would like you to participate.

When parents have had a child with Down Syndrome there is good information available, collected from many families, as to the chance of those parents having another affected child. You will probably already have received this information from your doctor or through the Genetics Clinic.

However, parents of a Down Syndrome child often wonder if their other normal children, or even their own brothers and sisters, may be more likely than other parents to have a similarly affected child. There has been a lot of difference of opinion as to this point. If there is any increase of risk for Down Syndrome children to this group of people it is so slight that we usually tell them that their risk is essentially the same as for any other parents in the same age group.

Recently a variation in the chromosomes, which is sometimes found in perfectly normal people, has been thought possibly to be associated with an increased risk for nondisjunction. This is the error in the formation of the egg or the sperm which is the cause of the extra chromosome in Down Syndrome. If this idea is true then it might be expected that other people in the family would also have the variant chromosome and perhaps it is only in these families that there is some increased risk for more distant relatives than the parents to have children with Down Syndrome.

As a first approach to this problem we thought it would be useful to look for the variant chromosome in the parents of the Down Syndrome child.

As your family is one which is known to the Genetics Clinic as having a child with Down Syndrome we would like to request your permission to obtain a blood sample from you (both parents if possible) for chromosome analysis.

In addition, we require some information on your family tree. To the best of your knowledge are there any additional members of your family, either living or deceased, with a genetic disorder (for example, Down Syndrome)? In defining the members of your family, this would include both close relatives (siblings, offspring, aunts, uncles, first cousins) and distant relatives (great-aunts, great-uncles, second cousins).

In due course we will be pleased to let you know the results although it should be emphasized that at the present time we do not know the true significance of this normal variation in the appearance of the chromosomes. Please feel free to contact me for any additional information you might wish on this project.

Yours sincerely,

Elizabeth Roberts

APPENDIX 2

The Explanatory Letter Given to Subjects in Test Group B

Dear

We are writing to you in order to explain a research project in which we would like you to participate.

When parents have had a child with Down Syndrome there is good information available, collected from many families, as to the chance of those parents having another affected child. You will probably already have received this information from your doctor or through the Genetics Clinic.

However, parents of a Down Syndrome child often wonder if their other normal children, or even their own brothers and sisters, may be more likely than other parents to have a similarly affected child. There has been a lot of difference of opinion as to this point. If there is any increase of risk for Down Syndrome children to this group of people it is so slight that we usually tell them that their risk is essentially the same as for any other parents in the same age group.

Recently a variation in the chromosomes, which is sometimes found in perfectly normal people, has been thought possibly to be associated with an increased risk for nondisjunction. This is the error in the formation of the egg or the sperm which is the cause of the extra chromosome in Down Syndrome. If this idea is true then it might be expected that other people in the family would also have the variant chromosome and perhaps it is only in these families that there is some increased risk for more distant relatives than the parents to have children with Down Syndrome.

As a first approach to this problem we thought it would be useful to look for the variant chromosome in the parents of the Down Syndrome child in families where there are two or more such children among fairly close relatives - cousins or closer. If it is found

then we would like to ask permission through you to examine the chromosomes from other members of the family.

As your family is one which is known to the Genetics Clinic as having more than one individual with Down Syndrome or another chromosome abnormality we would like to request your permission to obtain a blood sample from you for chromosome analysis.

In due course we will be pleased to let you know the results although it should be emphasized that at the present time we do not know the true significance of this normal variation in the appearance of the chromosomes. Please feel free to contact me for any additional information you might wish on this project.

Yours sincerely,

Elizabeth Roberts

APPENDIX 3

The Consent Form for the Collection of Blood Samples

TITLE OF RESEARCH PROJECT: DOUBLE NUCLEOLAR ORGANIZING REGIONS AS A RISK FACTOR FOR DOWN SYNDROME

INVESTIGATOR(S): Dr's. E.J. Ives, C.C. Lin and E.M. Roberts

Consent for collection of a blood sample for chromosome analysis in relation to the above project, a description of which is attached.

I have read the attached letter explaining the above project and am willing to provide a blood sample for chromosome analysis. I understand that if the chromosomal variant is found, the significance of this observation will remain unknown until further studies are made.

I acknowledge that the research procedures described on the attached form and of which I have a copy, have been explained to me and that any questions that I have asked have been answered to my satisfaction. I have been informed of the alternatives to participation in the study. I also understand the benefits (if any) of joining the research study. The possible risk and discomforts have been explained to me. I know that I may now, or in the future, ask any questions I have about the study or the research procedures. I have been assured that personal records relating to these experimental protocols will be kept confidential and that no information will be released or printed that would disclose personal identity without my permission.

I understand that I am free to withdraw from the study at any time. I further understand that if the study is not joined, or if there is withdrawal from it at any time, the quality of medical care will not be affected.

The person who may be contacted about the research is:
Dr. E.J. Ives or Elizabeth Roberts
Telephone No. (403) 432 - 4077

(Name)

(Signature of subject)

(Name)

(Signature of witness)

(Date)